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A STUDY OF PHARMACEUTICAL MEDICINES IN HUMAN PLASMA

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ABSTRACT

A study of sensitive and rapid chromatographic method was developed and validated for the simultaneous quantification of atenolol and chlorthalidone in human plasma using hydrochlorothiazide as internal standard (IS). The method utilized proteins precipitation with acetonitril as the only sample preparation involved prior to reverse phase-HPLC. The analytes were chromatographed on Shim-pack cyanopropyl column with isocratic elution with 10 mM KH2PO4 (pH 6.0) – methanol (70:30, v/v) at ambient temperature with flow rate of 1 mL min1 and UV detection at 225 nm. The chromatographic run time was less than 10 min for the mixture. The calibration curves were linear over the range of 0.1–10 lgmL1 . The method was validated in terms of accuracy, precision, absolute recovery, freeze–thaw stability, bench-top stability and re-injection reproducibility. The within- and between-day accuracy and precision were found to be within acceptable limits.

KEYWORDS: Pharmaceutical Medicines, Human Plasma, chromatographic method

INTRODUCTION

Atenolol is a b1-selective b-adrenergic receptor-blocking agent and clinically used for the treatment of hypertension. Like other antihypertensive drugs, atenolol lowers the systolic and diastolic blood pressure by 15–20% in a single drug treatment and reduces cardiovascular mortality. It is also used alone or in combination with other antihypertensive agents for the treatment of myocardial infraction, arrhythmias, angina and disorders arising from decreased circulation and vascular constriction, including migraine. Atenolol is rapidly but incompletely absorbedafter oral administration. It is excreted almost entirely as unchanged drug, 35–50% of an oral dose being excreted in the urine and 30–50% in the faeces in 24 h. After a single oral dose of 100 mg given to 12 subjects, peak plasma concentrations of 0.41–0.87 mg1 L (mean 0.6) were attained in about 3 h. Chlorthalidone is a diuretic drug widely used in hypertension therapy.

It does not appear to be significantly metabolized. After a single dose, about 25–40% is excreted in the urine as unchanged drug and about 1% is eliminated in the bile; the quantity excreted in the urine appears to be dose-dependent. During daily therapy, about 50% of the daily dose is excreted unchanged in the urine in 24 h and about 25% is eliminated in the faeces. After single oral doses of 50–75 mg given to 7 subjects, peak plasma concentrations of 0.14–0.26 mg/L were attained in 1– 3h. In recent years, pharmaceutical preparations containing both drugs have also been marketed, their combination having the advantage of providing greater therapeutic effects than with either drug alone or permitting once a day administration only, the two drugs not

interacting pharmacokinetically with each other or presenting synergic toxic effects. The two drugs are of limited metabolism and eliminated unchanged from human body. In open literature, several methods have been reported for the determination of atenolol in biological fluids that relied on HPLC with UV or fluorimetric detection, HPLC with MS detection, and adopting gas chromatographic techniques with an electron capture detector. On the other hand, chlorthalidone has been measured in biological fluid both by GC and nitrogen detection, HPLC using analogous columns and UV detection, or HPLC coupled with MS detector. The previously reported methods applied liquid-liquid extraction or solid phase extraction as the main technique and there is no method applied for protein precipitation technique for their determination in biological fluids. Atenolol and chlorthalidone mixture is officially listed in USP. To our knowledge, there are many methods that have been published for simultaneous determination of both drugs in pharmaceutical formulations like chemo metric-assisted spectrophotometric and HPLC with UV detection. These methods were not suitable for simultaneous determination of the proposed mixtures in biological fluids due to matrix interference. Recently our laboratory applied chromatographic technique for simultaneous determination of ternary mixtures for hypertension treatment containing the two studied drugs. This method failed to quantitate the studied mixture in plasma because of the short retention time of atenolol that interfered with plasma matrix. Additionally there are two methods that have been described for simultaneous determination of both drugs in human milk and plasma. These methods required long time for preparation of samples or relied on tedious liquid-liquid extraction procedure.

REVIEW OF LITERATURE

Challa B. R. et al (2012) explain method for medicines by using liquid-liquid extraction (LLE) technique. Zorbax, SB C18, mm, 3.5 µm particle size analytical column was used for analysis. Mobile phase was made with combination of 10 mM ammonium acetate buffer (pH 5): methanol (10:90, v/v). The ion transitions for medicines (m/z 427.3 → 147.3) and IS (m/z 431.4 → 151.2) were monitored on MRM mode at positive ion mode by using LC-MS/MS. The linearity range was 10.00– 20000.00 pg/ml.

Andrade A. S. et al (2011) validated method with a C8 (150 × 4.6 mm, 5 µm) column. The wavelength was selected 205 nm. The rate of flow was 1.1 ml/min. The mobile phase consisted with acetonitrile (MeCN) and orthophosphoric acid (OPA) 0.01% (v/v) with pH 3.0 in a ratio 28:72 and column oven temperature was 25 ± 1°C. The analysis were performed by using forced decomposition techniques and quantified by LC-MS/MS.

Himabindu V. et al (2008) explain that analysis done by using immobilized cellulose based chiral stationary phase chromatography. Mobile phase was consisted as n-hexane:ethanol:diethylamine (50:50:0.3, v/v/v). The rate of flow was 1.0 ml per minute. The elution time was ~15 min. The LLOQ for the (R)-enantiomer 0.07 µg. Injection volume was 10 µl.

Krishnaiah Ch. et al (2013) achieved chromatographic separation done by using Acquity UPLC BEH C18 column (100 x 2.1 mm, 1.7 µm). Flow rate was 0.3 ml/min and detected at 210 nm. The molecular ion peaks at mass to charge ratio 428.20, 425.20 and 281.30 for the acid (Impurity 4), oxidized (Impurity 6) and N-dealkylated (Impurity1) forms of darifenacin respectively.

Sridharan D. et al (2011) carried chromatographic separation on an Inertsil ODS3V (250 X 4.6mm, i.d., 5µ) column. The mobile phase was consisted with gradient composition of potassium dihydrogenortho phosphate buffer and acetonitrile. The rate of flow was 1.0 ml per minute. The UV detection wavelength was selected 220 nm. The time of retention for darifenacinhydrobromide is about 4.8 minutes. The percentage recovery for darifenacinhydrobromide was found to be 98 to 102%. The detector response is linear from 37.91 to 113.73µg/ml.

Ganeswar M. et al (2012) achieved chromatographic separation done by using Enable C18G column (250mm × 4.6mm, 5µm). The mobile phase was made up of methanol: 10mM TBAHS (60:40, v/v). rate of flow was 1.2 ml/min detected by photo diode array at 285 nm. Linearity range was 1.0–200 µg/ml.

Ashwinee K. S. et al (2011) has developed method for darifenacinhydrobromide absorbance at 286 nm. The linearity range was 10 to 100 µg/ml. The solutions were found to be stable for the various concentrations ranging from 10-100% at 30 and 60 minutes.

Aswani D. C., et al (2011) explained the method for darifenacinhydrobromide determination by using UV as detector. The Linearity range was 20.00-46.50µg/ml.

Bhavin N. P. et al (2011) has developed bioanalytical method for medicines in plasma by using niacin as IS. Waters Oasis MCX cartridge was used for solid phase extraction. Analysis done by using Peerless Basic C18 (100 mm × 4.6 mm, 3 µm) column.Detection done by positive ion and MRM acquisition mode. The m/z for cycloserine and niacin were at 103.1 → 75.0 and 124.1 → 80.1 respectively. The linearity range was 0.20–30.00 µg/ml.

Nageswara R. P. et al (2013) described bioanalytical method for medicines in plasma by using carbamazepine as IS. Protein precipitation used as Extraction method. The chromatographic column was C18 and mobile phase consist with acetonitrile–0.5% formic acid buffer (60:40, v/v). Rate of flow was set 0.8 mL per minute. The calibration curve range was 50–15,000 ng/ml. Run time for analysis was 2.5 min.

Ashish M. et al (2012) has developed bioanalytical method for the determination of medicines in plasma. Acyclovir is used as internal standard. Water Oasis MCX 1cc/30mg cartridge was used for solid phase extraction. The chromatographic column was Inertsil ODS-3V (4.6 × 250 mm, 5µ). The mobile phase was prepared by the mixture of milli-Q water: acetonitrile: formic acid (30:70:0.3 v/v). The run time was 2.6 min. The product ion transition were monitored in MRM and positive ion mode. The linearity range was 0.200 to 16.000 µg/ml.

Andy D. et al (2012) evaluated the analysis of cycloserine. In this method normal phase used with silica hydride-based stationary phase and detected by mass spectrometry. Analyses done by using gradient as well as isocratic conditions.

Ann V. S. et al (2008) described LC–UV method by using Hypersil BDS column; 25 cm × 4.6 mm column kept at 45 °C. The mobile phases was prepared by using mixture of acetonitrile: 20 mM sodium octane sulphonate: 0.2 M potassium dihydrogen phosphate buffer pH 2.8: water: (4:70:10:16 v/v/v/v) was denoted as A and B was consisted of (17:70:10:3 v/v/v/v). By using A & B gradient

mode where used for chromatographic separation.

Chen Y. et al (2012) described HPLC-ESI-MS/MS method with m/z 178.0/ 90.9. XTerra MS C18, 150 mm × 2.1 mm, 5 µm column was used for determination. Mobile phase was consisted of 20 mm ammonium formate and 0.5% of formic acid into methanol (25:75, v/v) with flow rate 0.3 mL.

Fu Q. et al (2009) has developed HPLC-MS method by using human plasma as biological matrix. Plasma sample were prepared by using 100 mL/ L perchloric acid. Calibration curve range was 0.2 to 200.0 µg/ mL

PLASMA-DERIVED MEDICINES: ACCESS AND USAGE ISSUES

Although the first plasma-derived medicines were anti-toxins, raised in horses against pathogens (diphtheria, tetanus), the inception of plasma protein therapy occurred when Edwin Cohn developed his fractional ethanol precipitation scheme to isolate a stable albumin solution for the treatment of battle field injury and blood loss2. The Cohn scheme yielded albumin as a final product while producing, among others, therapeutically useful fractions of fibrinogen and immunoglobulins which could not be employed widely because of safety issues. Following initial safety problems, albumin gained widespread medical acceptance because of its dramatic effectiveness reported in victims of shock3, and was the plasma industry's staple product until the 1970s. Its position as a safe and effective plasma expander went unchallenged up to the 1990s, when the introduction of cheaper synthetic colloids and a Cochrane meta-analysis4 threw doubts on its use, which were subsequently dispelled through clinical trials5.

While studies showed that careful plasma processing of Cohn's Fraction I could yield a product which was therapeutically useful in haemophiliaA6, it took Judith Pool's widespread adoption of cryoprecipitate from blood bank plasma7 to result in the next milestone in the history of plasma protein therapies. Pool's technique was rapidly adapted for large-scale fractionation without affecting the Cohn method8 and resulted in the first industrial scale production of haemophilia therapy. The capacity to treat a previously life-limiting disease effectively made the manufacture of factor VIII (FVIII) concentrate the driver for the plasma industry in the 1970s, usurping albumin's historical position. The revolution this produced in the life of haemophiliacs cannot be underestimated, just as the effects, on patients and industry alike, of viral transmission by the products cannot be underappreciated, although in the heady days of the 1970s this risk of this transmission was under recognised.

While industry hastened to introduce enhanced safety measures, particularly viral inactivation which by the mid 1980s had made haemophilia products safe, an effect of this tragedy was the rapid development of recombinant FVIII concentrates, once the F8 gene had been cloned in 19849. The results of clinical trials, published in 198910, rapidly led to widespread acceptance of this therapy to its current position as the dominant haemophilia treatment modality in many countries of the developed world, including the United States, the United Kingdom, Canada and Australia, and greatly increased the market and the availability of FVIII, allowing interventions such as prophylaxis and tolerisation. In some other, mostly European countries, plasma-derived FVIII has retained a strong presence, due, primarily, to the continuing debate regarding the different capacity of different FVIII products to result in inhibitors to

FVIII11. This development would have had a profound effect on the economic, and indeed, the viability of the industry, but other developments in the field of immunotherapy obviated it.

Cohn's original method allowed the harvesting of immunoglobulin (Ig) fractions which could be concentrated into solutions and used to treat patients with Ig deficiencies12. In addition, Ig solutions from the plasma of donors immunised to specific antigens could be used for the treatment or prophylaxis of various diseases; the use of the Rh Ig fraction is the most famous of these applications13. However, early clinical observations that intravenous administration of Ig solutions led to severe reactions meant that Ig administration was limited to the intramuscular route, limiting dosage and patients' comfort. Efforts to address this problem led to several imperfect intravenously administrable Ig products, in which measures, such as enzymatic digestion of the entities causing reactions, principally aggregates of Ig, formed during fractionation, also damaged the Ig molecule, limiting its half-life in vivo.

Table 1 Main indication for administration of intravenous immunoglobulin

Indication	Dosage
Antibody-associated immune deficiencies	0.4 g/kg/4 weeks
Chronic inflammatory demyelinating polyneuropathy	2 g/kg in 2 to 5 divided doses
Multifocal motor neuropathy	2 g/kg in 2 to 5 divided doses
Guillain-Barré syndrome	2 g/kg in 2 to 5 divided doses
Idiopathic (autoimmune) thrombocytopenic purpura in adults	1 to 2 g/kg as single or divided dose
Kawasaki disease	2 g/kg in a single dose

The efforts to overcome these difficulties were spurred on by clinical findings that intravenous administration of large doses of Ig was helpful in ameliorating a number of autoimmune pathologies, such as immune thrombocytopenic purpura (ITP). Once well-tolerated and molecularly intact intravenous immmunoglobulins (IVIg) were produced, the efficacy of the product in a wide range of these pathologies continued to be demonstrated15. In addition, the capacity to deliver large doses intravenously allowed more effective treatment of immune deficient states16. These combined features led to IVIg becoming the predominant plasma protein therapy, and the industry's driver, by the 1990s, a position it holds today. Table 1 lists the main approved indications for IVIg and their recommended dosages.

Despite this, the three generations of staple plasma-derived medicines-albumin, FVIII and IVIg- form part of every manufacturer's portfolio and are claimed to be crucial in maintaining the industry's viability17. A number of less economically important but therapeutically crucial additional products have also evolved over the years, including therapies for other bleeding disorders, for congenital deficiencies of the plasma proteins

and for treating injury18. Table 2 summarises some features of these "second tier" products. Significant regional variations are found in the usage of these products, resulting from differences in clinical practice, regulatory approval and availability in specific areas.

Table 2 Second tier plasma products - usage in different regions

Product	Units	North America	South America	Europe	Asia and Pacific
Fibrin sealant	mL (×1000)	15	10	932	875
Prothrombin complex concentrate	International Biological Units ($\times 10^6$)	7	23.1	306	16.9
Alpha 1 anti-trypsin	Vials (×1000)	470	/	166	/
Anti-thrombin III	International Biological Units ($\times 10^6$)	15.0	0.8	289.9	349.6

CURRENT ACCESS ISSUES REGARDING PLASMA-DERIVED MEDICINES

Reimbursement issues

Plasma-derived medicines are the products of expensive technologies using a complex raw material, human plasma, which has to be procured from large numbers of blood or plasma donors. The complex technologies, the price of the raw material and the multiple safety measures contribute to the cost of these products. With some notable exceptions, the target patient populations are small, suffer from rare disorders and the indications are often classifiable as orphan indications. Together, these factors contribute to the relatively high cost of plasma protein therapies.As health budgets have been subjected to increasing pressures, the funding, through reimbursement pathways from private and public payers, has come under scrutiny. This has led to plasma protein therapies being drawn into the landscape of Health Technology Assessments (HTA), including the possible application of cost-utility analysis (CUA) in the allocation of reimbursement funds. Given the high cost of the therapies and the relatively nascent nature of many of the indications for their use, application of these tools of health economics can lead to restriction of the supply of products for patients in genuine need of them. This can occur if appropriate attention to all the factors contributing to clinical efficacy and quality of life are not considered. These issues will be discussed in relation to two "staple" products, FVIII and IgG.

Access to the raw material

Unlike the situation for most pharmaceuticals, the costliest component of the manufacture of plasma protein therapies is the raw material, plasma. In well-managed and economically unrestricted environments, there is seldom a problem in assuring product supply, as compensation of donors ensures a constant supply of raw material which, in countries subject to resourced regulatory agencies, is entirely safe. Restrictions to industry's capacity to access donors are, therefore, an impediment to access to plasma protein therapy. Driven by considerations which include WHO resolutions and underpinned by economic factors, including the protection of domestic blood systems, some countries

declare a policy of "self-sufficiency" in plasma-derived medicines. In practice, this policy is usually a policy of "non-importation", as a particular form of trade barrier, as there is little "sufficiency" in the availability of plasma products in these countries. In many cases, the self-sufficiency countries include those which supply the lowest amounts of essential therapies. Interestingly, this is not a function of economic status-rich countries such as Japan may have a low consumption of some products such as FVIII and Ig through restricting the use of non-domestic products, while some less economically developed countries, such as Hungary, show higher than expected levels of consumption through allowing access. Global sufficiency of plasma protein therapies requires unrestricted collection of plasma according to regulatory and clinical requirements, free access of traded products across borders and usage practices based on clinical needs and evidence. A policy that results in restricting access to treatment for the population of vulnerable plasma protein recipients is ethically dubious at best.

USAGE OF PLASMA PROTEIN THERAPIES

Blood-derived therapies have been sheltered, historically, from many of the requirements which other medicines have for the provision of evidence of efficacy. This is still predominantly the situation for fresh blood components, which, while subject to scrutiny for safety and quality through the application of standards and good manufacturing practices, are not required to show efficacy in most regulatory frameworks. This state of affairs may change in the face of accruing evidence that many of the traditional assumptions regarding issues of efficacy, dosage, etc. for red cells, platelets and plasma are questionable. In contrast, the suppliers of plasma-derived medicines have had to satisfy therapeutic claims since the absorption of these products into mainstream medicinal regulation such as found in North America and the European Union.

In addition, the application of the tenets of the evidence-based medicine (EBM) movement has started to engage the landscape of plasma-derived medicines, in ways which influence access to treatments over and above the need to satisfy regulatory requirements. Structuring haemophilia treatment and Ig use according to clinical guidelines based on the EBM "hierarchy" for example, has facilitated access in several countries and, somewhat unexpectedly, has been accompanied by substantially increased product usage in some countries. These benefits of the application of EBM are offset, potentially, by the strict interpretation of the definition of "best quality" evidence as requiring randomised clinical trials. The epistemological problems concerning randomised clinical trials have been discussedand lead to questions regarding claims to superiority of such trials as the sole tool for generating evidence. With particular relevance to plasma-derived medicines in small populations of patients, well-conducted observational studies are important if consideration of the majority of clinical experience with rare disorders is to be drawn upon. As a recent example, the dismissal of the efficacy of alpha 1 anti-trypsin augmentation in patients with alpha 1 anti-trypsin deficiency through limiting a meta-analysis to two small randomised clinical trials performed by the same clinical group in one location compels critical scrutiny of the Cochrane Review which performed this analysis. Other forms of evidence, more suited to small populations of patients, indicate the value of this treatment. Occasional comments on the cost of treatments (for example, those in) within these assessments result in the

perception of a commitment to cost-minimisation rather than to generating evidence.

IMPORTANT IN DRUG STUDIES AND USAGES OF HPLC

Often when compound exist in different isomeric forms it is a mixture of isomers. Alternatively there may be a need to monitor samples of one isomer to ensure it is purity. In both cases it is important to be able to separate isomers using liquid chromatography. This is especially important for drug substances, since isomers will almost certainly have different pharmacological and sometimes toxicological properties. Enantiomers are types of isomers that have aroused a great interest when the drastic teratogenic effects of the drug thalidomide (children born by mothers who had been taking thalidomide were born with truncated limbs) were attributed to one of the two enentiomers present in the drug substance. Enantiomers have identical physiochemical properties and therefore cannot be separated on conventional chromatography. Thus in the liquid chromatography separation of enantiomers it is necessary to use a "chiral selector". This may take the form of a chiral derivation agent, a chiral mobile phase additive or a chiral stationary phase. Typical common diastereomer formations the types of derivation reagents that may be used are shown of Fig. 1.

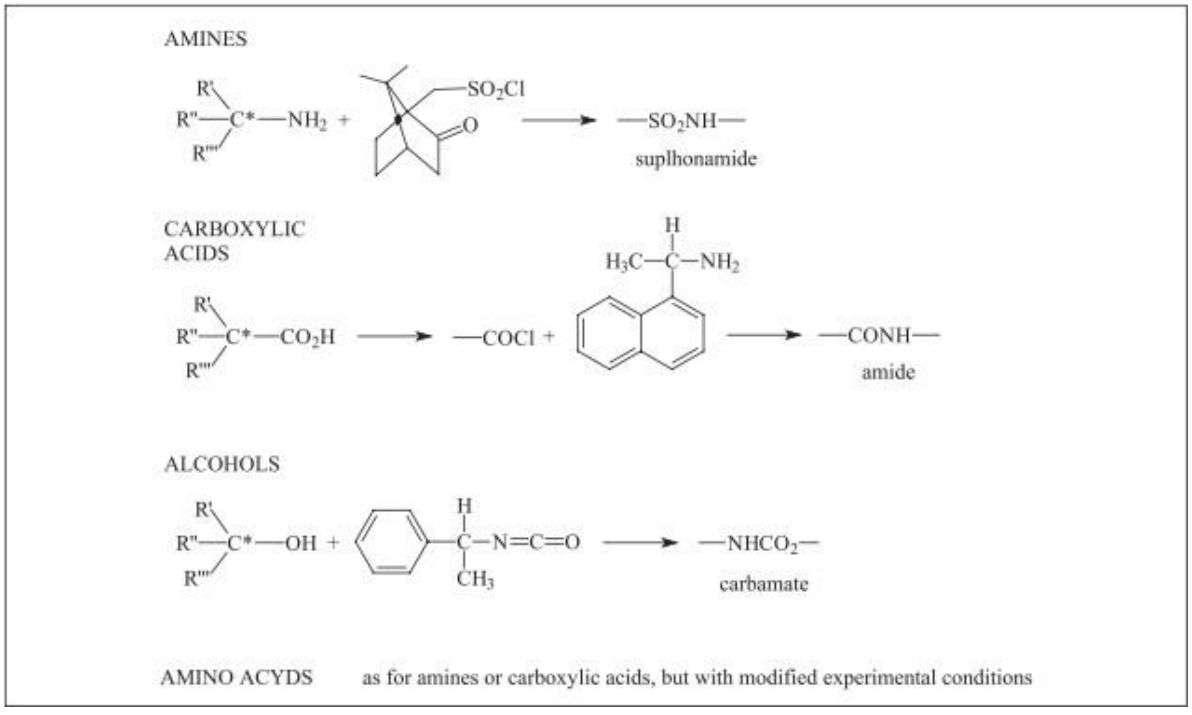

Figure 1. Typical common diastereomer formations

The products formed are diastereoisomers. These compounds, which possess two "chiral centres", have different physicochemical properties and are therefore separable by liquid chromatography on conventional "achiral" liquid chromatography columns. For a number of reasons it is usually preferable to use a chiral stationary phase6) rather than a

chiral derivation agent. A chiral stationary phase will normally be preferred to a chiral mobile phase additive, partly becausethe use of a chiral selector as a mobile phase additive will lead to much higher consumption of an often expensive compound. Entatiomerically pure chiral compounds occur in nature and are readily available fairly cheap. These compounds therefore form the basis of most commercially available chiral stationary phase. Most commonly used are proteins, cyclodextrins, derivatised polysaccharides and derivatised amino acids. Liquid chromatography techniques are very convenient for the study of the interactions between small molecules and large molecules, in particular to study drug-protein binding. Some investigator have used immobilised human serum albumin phase to study the interaction of drugs such as benzodiazepines, warfarin, ibuprofen and others. The use of this phase as a model of interactions that take place in vivo may be taken a stage further. By adding a drug to the mobile phase it is possible to study how the interaction of one drug with human serum albumin is affected by the presence of another.

IMPORTANT PART IN QUALITY CONTROL OF DRUGS

The study of stability in pharmaceuticals is vital because of the need to avoid the potentially toxic degradation products. In such studies it is necessary to demonstrate that the drug content of the formulation has not changed with time. Also if degradation does take place it will be necessary to identify and quantify the degradation products. A good illustration of this is the liquid chromatography conditions developed for the determination of pilocarpine in ophthalmic solutions. The identical conditions are used for the determination of pilocarpine degradation products i. e. isopilocarpine and pilocarpic acid. 7)Almost in all the laboratories for the quality control of drug the official methods for the assay of antibiotics are microbiological measurements. In order to provide it a separate laboratory for microbiological investigation has to be establishing with specialized staff (microbiologist). Nowa day's high performance liquid chromatography technique is going to be used for the quantitative determination of antibiotics. That is a great advantage in the field on quality control of drugs.

PREPARATION OF STANDARD AND QUALITY CONTROL

Stock standard solutions of the studied drugs in the two mixtures (1 mg mL1) were separately prepared in methanol. Working solutions were prepared by appropriate dilution in methanol just before use. All solutions were stored in darkness at 4 C. Volumes of 20 lL of the preparing working solutions and 20 lL of IS solution (150 lg mL1) were added to 960 lL of drug-free human plasma to obtain drug concentration levels of 0.1, 0.2, 0.6, 1.0, 2.0, 5.0, 7.0, and 10 lg mL1 . Quality control (QC) samples were prepared separately and pooled at three different concentration levels (0.1, 1, 10.0 lgmL1) as low, medium and high, respectively. The samples were stored in a freezer at 20 C until analysis. A calibration curve was constructed from a blank sample (a plasma sample processedwithout the IS), a zero sample (a plasma processed with the IS) and eight non-zero samples covering the total range of 0.1–10.0 lg mL1 , including the LOQ. Calibration curves were generated using the analytes to IS peak area ratios by leastsquares linear regression.

CONCLUSION

The method developed is a simple, rapid, accurate, and reliable procedure for the analysis of atenolol and chlorthalidone in human plasma, meeting all requirements for the validation of an analytical methodology. It is adequate to monitor patients receiving therapeutic dosage of the drugs.Although every societal grouping claims its issues are unique, there are several distinctive features which differentiate plasma protein therapies from mainstream treatments produced for large populations of patients. There is no reason for these differences to constitute insurmountable problems to the provision and use of these therapies, as long as the need for adaption of standard reimbursement and evidence processes is accepted. Recognising the clear benefits to chronically diseased patients needing these treatment should be foremost in the minds of the relevant decision-makers. Finally, it must be recognised that the pressures on access in the developed countries, substantial as they are in the current financial climate, still fade into insignificance when compared to those in the less developed world.

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