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Article

On-line Sample Cleanup and Enrichment Chromatographic Technique for the Determination of Ambroxol in Human Serum

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A sensitive and efficient on-line clean up and pre-concentration method has been developed using column-switching technique and protein-coated μ-Bondapak CN silica pre-column for quantification of ambroxol (AM) in human serum. The method is performed by direct injection of serum sample onto a protein-coated μ-Bondapak CN silica pre-column, where AM is pre-concentrated and retained, while proteins and very polar constituents are washed to waste using a phosphate buffer saline (pH 7.4). The retained analyte on the pre-column is directed onto a C_{18} analytical column for separation, with a mobile phase consisting of a mixture of methanol and distilled deionized water (containing 1% triethylamine adjusted to pH 3.5 with ortho-phosphoric acid) in the ratio of 50:50 (v/v). Detection is performed at 254 nm. The calibration curve is linear over the concentration range of 12–120 ng/mL ($r^2 = 0.9995$). The recovery, selectivity, linearity, precision, and accuracy of the method are convenient for pharmacokinetic studies or routine assays.

Introduction

Ambroxol (AM) is a drug with potent mucolytic activity and used as an expectorant and bronchosecretolytic in therapeutics (1, 2). AM stimulates the transportation of the viscous secretion in the respiratory organs and reduces the stagnation of the secretion.

Several analytical methods for measuring plasma levels of AM either individually or in combination with other drugs have been reported. Most of these methods were based on high-performance liquid chromatography (HPLC) and utilized UV or amperometric detections (1, 3–6). Also, capillary electrophoresis with fluorescence detection has been reported (7). These HPLC procedures involve pre-treatment of the AM biological samples with off-line methods, typically using liquid–liquid extraction (LLE) to obtain maximum recovery and minimum or no interference of biological fluids. The main drawback of these HPLC protocols were time-consuming, labor-intensive, and/or rather unselective sample cleanup steps. In addition, the reported lower limit of quantification (LOQ) for AM was not nearly low enough to adequately define the concentration-time profiles in these studies. These difficulties encouraged the development of hyphenated techniques combining HPLC with tandem mass spectrometry (HPLC–MS–MS) for the identification and quantification of AM in human plasma (8–11). However, the sample cleanup procedures limited the ultimate performance of these methods, especially with regard to ruggedness and reliability. Also, they were not widely applicable due to their expensive cost for the common user. AM was also determined in human plasma using gas chromatography (12, 13). Most of these methods were difficult and time-consuming and required a derivatization and LLE steps.

Column-switching systems have greatly contributed to the direct determination of drugs in biological fluids (14). The pre-columns packed with conventional reversed-phase materials were, however, unstable and with a relatively short life span when a large volume of serum was applied. The addressed problem was due to the fact that the column packing materials were not designed to tolerate the direct injection of protein-containing matrices. A coupling protein-coated μ-Bondapak CN silica pre-column to a conventional C_{18} analytical column will be a promising combination, because the protein-coated pre-column will deal with protein-containing samples more effectively. This pre-column has reversed-phase characteristics for small hydrophobic molecules and loses absorbability for serum proteins (15). In addition, it can be packed simply and economically in the laboratory, thereby lowering the cost of AM analysis by HPLC.

The aim of this work was to expand the automation in sample preparation by developing direct injection HPLC-UV technique as a low-cost and facile approach for the determination of AM in serum samples. The described method avoided the use of large amounts of organic solvents in extraction and pre-concentration steps and, additionally, offered advantages such as: (*i*) incorporating sample extraction, concentration and separation into a single procedure; (*ii*) increasing sensitivity and selectivity; (*iii*) lowering environmental toxicity; (*iv*) and improving safety.

Experimental

Instrumentation

The HPLC (Shimadzu, Kyoto, Japan) apparatus, illustrated in Figure 1, consisted of two model series LC-10 ADVP pumps; one used to load the sample onto the pre-column and the other to flush the analyte from the pre-column to the analytical column. The system was equipped with two columns: one was short protein-coated μ-Bondapak CN silica, (10 mm × 4.6 mm i.d., 20 μm particle size) and the other was an analytical column of ZORBAX Eclipse XDB-C_{18} (150 × 4.6 mm i.d., 5 μm particle size from Agilent Technologies). A model 7725i sample injection valve (with a 250-μL sample loop) and a model 7000 flow direction column switching valve were applied to load the samples and facilitate the flushing of the analyte from the pre-column onto the analytical column, respectively (Rheodyne, Berkeley, California). An SPD-10AVP UV-VIS detector was used for the detection of AM at 254 nm. Data acquisition was performed on class-VP software. All determinations were performed at ambient temperature.

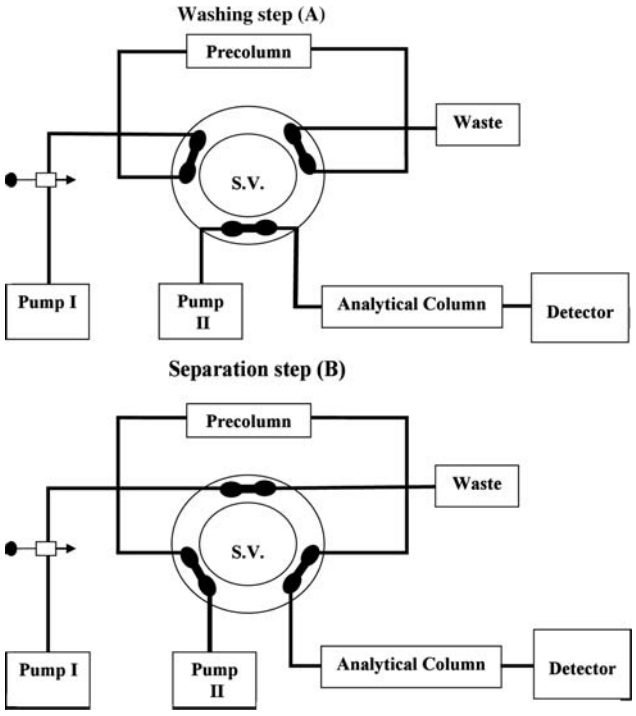

Figure 1. Scheme of the HPLC–integrated sample preparation. (A) Shows the system in initial position, ready for a sample injection and washing step; (B) Displays the separation step: the pre-column is connected with the analytical column via a 6-port switching valve (SV).

Materials and reagents

Pharmaceutical grade of AM (Ven Petrochem and Pharma, India) was used and certified to contain 99.8% AM. Disodium hydrogen phosphate, potassium dihydrogen phosphate, ethylene diamine tetra acetic acid disodium salt, sodium chloride, potassium chloride, sodium hydroxide, ammonium acetate (Sigma-Aldrich, Inc, St. Louis, MO) phosphoric acid, and triethylamine (BDH Laboratory Supplies Poole, England) were of analytical grade. The acetonitrile and methanol used were of HPLC grade (E. Merck AG, Darmstadt, Germany). The bovine serum albumin (BSA) of fraction V powder was obtained from Sigma. The rabbit dialyzed plasma (plasma protein fraction) was prepared by 3 times dialysis of the rabbit plasma in a visking tube against 100 volume of phosphate buffered saline, pH 7.4, at 25°C for 4 h. The μ-Bondapak CN silica was from Waters Associates (Milford, MA).

Mobile phases

Two different mobile phases were employed in the assay procedure. One was a phosphate buffer saline (pH 7.4) (MI), which was used to deliver the sample to the extraction protein-coated pre-column for cleanup and enrichment of AM from the serum. The second mobile phase (MII) was a mixture of methanol–distilled deionized water (containing 1% triethylamine adjusted to pH 3.5 with ortho-phosphoric acid) in the ratio of 50:50 (v/v), and it was introduced as an analytical mobile phase. Following their preparations, the mobile phases were passed through 0.45 μm filters (Millipore, Bedford, MA) and degassed using a sonicator for 5 min.

Phosphate buffer saline, pH 7.4 (MI)

The phosphate-buffered saline stock solution contains 80 g sodium chloride, 2 g potassium chloride, 11.5 g disodium hydrogen phosphate, 2 g potassium dihydrogen phosphate, and 20 mL 0.1 M ethylenediamine tetra acetic acid disodium salt per liter. The pH was adjusted to 7.4 with 1 M sodium hydroxide solution (MI).

Pre-column preparation

The μ-Bondapak CN silica pre-column was equilibrated with phosphate buffer saline (pH 7.4). Then, 50 μL of 6% BSA solution were injected under the flow rate of 2 mL/min and at 25°C. The pre-column was also equilibrated with 0.1 M phosphate solution (pH 3.0), and 50 μL of 6% BSA were injected again. The pre-column was then washed with methanol. The procedures mentioned herein (BSA saturation and methanol washing) were repeated several times. The column thus obtained is called the BSA-coated pre-column. The protein-coated pre-column was prepared from the BSA-coated column as follows: 50 μL of the rabbit dialyzed plasma were injected with 0.1 M phosphate solution (pH 3.0), and the column was washed with methanol. These steps were important to immobilize the absorbed proteins on the outside surface of the porous packing materials. The interaction of BSA with the plasma proteins may be important for both protein-immobilization, and also to lose the affinity for natural plasma proteins. Thus, the procedures mentioned herein (plasma protein saturation and methanol washing) were repeated several times.

Chromatographic procedure

The main steps of the process are schematically represented in Figure 1. In the equilibrium phase (position A), preceding the injection of the serum sample, MI was brought onto the pre-column by pump I at a flow rate of 1 mL/min for equilibration; meanwhile, pump II equilibrated the analytical column with MII at a flow rate of 1 mL/min. Then, 250 μL of the serum were injected into the sampling valve and transferred to the pre-column, which was then flushed with 2 mL of MI to remove any large protein fragments and other hydrophilic components. The switching valve was then rotated to the elution mode. In this position (B), the retained compound on the pre-column was directed onto the analytical column by MII. After 2 min, the switching valve was switched again (position A), and while the compound of interest was chromatographed on the analytical column, the pre-column was re-equilibrated with MI to be ready for the next injection.

Standard and stock solutions

A primary stock solution of AM was prepared by dissolving the appropriate amount, which was accurately weighed in methanol and successfully diluted with the same solvent to achieve the concentration of 120 μg/mL. From the primary stock solution, seven mixed working standard solutions of AM over the

concentration range of 0.12–1.2 μg/mL were prepared in methanol. Then, each working standard solution was diluted 10-fold into drug-free human serum to obtain the concentration range of 12–120 ng/mL. The spiked serum samples were stored frozen at −20°C until used.

Specificity

Pooled blank serum samples were used to determine whether endogenous matrix constituents co-eluted with AM. Blank samples were obtained from six different healthy donors and were individually analyzed to determine if there are any interfering peaks around the retention time of the analytes.

Recovery, precision and accuracy

Intra- and inter-day precision and accuracy were established at three concentration levels (15, 40, and 100 ng/mL) and five replicate analyses of each sample were analyzed on a single assay day (intra-) and on five consecutive days (inter-day). The recovery of AM from the serum was calculated by comparing the peak area measured for the spiked serum samples treated according to the described procedure to that of the aqueous solution with the same concentration of AM. The precision was expressed as the coefficient of variation (CV), and the accuracy was expressed as the relative errors (RE) of the mean measured concentration. The RE was evaluated by back-calculation and expressed as the percent deviation between the amount found and amount added according to the following:

$$\text{RE} = [(\text{conc. found} - \text{conc. added})/\text{conc. added}] \times 100$$

Results and Discussion

Cleanup, enrichment, and separation conditions

In the present study, samples were fractionated into a sample matrix and the analyte by the use of the protein-coated pre-column. When the serum sample was injected into the extraction pre-column, proteins were readily size-excluded without adsorption to the external surface, while a small drug molecule could penetrate into the pore and be retained on the internal surface. In this way, the packing material provided a direct extraction base, fully automated on-column enrichment, and subsequent analytical separation of low-molecular target compound from untreated serum samples. In order to create enough selectivity in the system and to get high recoveries of AM in the extraction and separation steps, it was necessary to optimize the extraction and the analytical mobile phase compositions, as well as the switching time of the valve.

MI must have the possibility for regulating the retention of AM and to separate it from serum matrix components. Also, MI should be compatible with the serum (i.e., serum proteins should be kept in solution). In this study, a short protein-coated μ-Bondapak CN silica pre-column was used for the enrichment and cleanup of the AM from the serum sample. The selected condition for loading the 250 μL serum sample onto the protein-coated μ-Bondapak CN silica pre-column was a phosphate buffer saline (pH 7.4) (MI) at the flow rate of 1 mL/min for 2 min. At this time point, most of the sample matrix was eluted from the pre-column; however, AM was still retained. AM was then selectively transferred via a switching valve onto a ZORBAX Eclipse XDB-C_{18} analytical column for further separation and quantification. In the transfer step, the adsorbed analyte was transferred from the pre-column to the analytical column by MII. Therefore, the composition of MII was also chosen in a way that enough selectivity could be created in the final separation of AM. For this reason, the influence of pH, triethylamine, and methanol concentration on the retention time of AM has been studied. A mobile phase consisting of a mixture of methanol and distilled deionized water (containing 1% triethylamine, adjusted to pH 3.5 with orthophosphoric acid) in the ratio 50:50 (v/v) gave a very good separation between the AM and endogenous components of serum matrix (Figure 2). Triethylamine was used as a peak modifier. The average retention time ± standard deviation (SD) for AM was found to be 4.74 min ± 0.02, for 10 replicates. The whole chromatographic process lasted for 6.74 min. The peak obtained was sharp and has a clear base line separation. Figure 2 shows the typical chromatograms for the determination of AM in a human serum samples from a subject 6 h after the administration of a single 30 mg dose ambroxol HCl tablet. No significant interferences from endogenous components in serum were observed.

Breakthrough study of protein-coated pre-column

For optimal pre-column conditions, with respect to band broadening, the retention of AM should be high at the enrichment mode and low at the elution mode (i.e., the pre-column hydrophobicity should be as low as possible but still completely enrich AM). A breakthrough study was performed by injection of the spiked serum sample containing AM onto the protein-coated pre-column, by means of MI. An increasing volume of MI was pumped through the pre-column before the valve was switched to position (B) and AM was brought to the

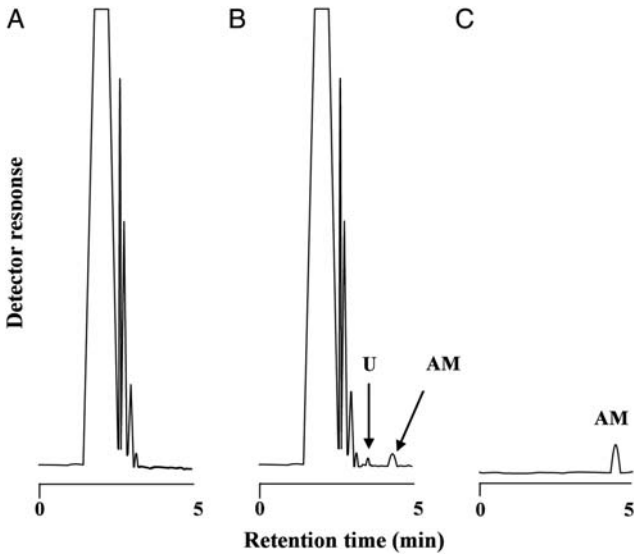

Figure 2. Representative chromatograms for the determination of AM. A: Blank drug-free human serum; B: Serum sample from a subject 6 h after the administration of a single 30 mg dose AM tablet, in which the concentrations was found to be 26.4 ng/mL; C: standard AM (60 ng/mL); U: unknown.

separation column with MII. With up to 10 mL of MI pumped through the pre-column, the recovery of AM was greater than 92%, which clearly showed that there was no risk of losing the drug during the cleanup step.

Selection of packing materials for protein-coated precolumn

The insertion of a pre-column in an HPLC system is very useful for the cleanup of as well as the enrichment from biological samples. In the present investigation, μ-Bondapak CN silica was chosen to be a suitable packing materials for preparation of easily replaceable protein-coated pre-column. The pre-column was prepared in the laboratory by immobilization of BSA, followed by rabbit dialyzed plasma proteins on the μ-Bondapak CN silica pre-column. The results indicated that the coated (immobilized) proteins were mainly adsorbed on the outside surface of porous μ-Bondapak CN silica, and the inside surface was not coated with proteins owing to the molecular sieve effect of small pores. This pre-column was a concern, as AM was not eluted from the pre-column even with 2 mL of MI. Elution of AM from the pre-column was too fast and, consequently, led to a narrower peak when using MII. A fast transferring time [the time needed for the delivery of AM from the pre-column into the analytical column (2 min)] with MII, which guaranteed peak compression, is crucial in avoiding dispersion and peak broadening in the pre-column that meant higher efficiency of the total separating system. The developed method has advantages in that the protein-coated pre-column has reversed-phase characteristics for small hydrophobic molecules and loses absorbability for the serum proteins. Also, it can be packed simply and economically in the laboratory, thereby lowering the cost of AM analysis by HPLC.

Selection of injection volume

Enrichment of AM at very low (ng/mL) levels is necessary to detect it at therapeutically levels when using HPLC-UV. Therefore, choosing an appropriate sample size has a large effect on the ability to detect and quantify AM in human serum. A protein-coated μ-Bondapak CN silica pre-column (10 mm × 4.6 mm i.d., 20 μm particle size) was used for the sample cleanup and enrichment and was combined with a C_{18} analytical column for the final separation. Different injection volumes (50–500 μL) were tested to introduce the decreasing concentration of AM. Different sample loops, ranging from 50 to 500 μL, were filled with serum samples when the switching valve was at position A. Then, MI carried the sample through the loop to the pre-column, where the AM was trapped, while the proteins and hydrophilic endogenous compounds, on the other hand, were excluded to waste. The loading capacity of the pre-column was found to have a significant influence upon recovery when a large sample volume was injected. It was found that the pre-column could tolerate large volumes of AM standard solution up to 500 μL. However, the same injected volume of serum samples (500 μL) was found to provide extremely high background signals that interfered with the detection of AM by means of UV monitoring. This can be explained by the presence of a large excess of early-eluting interferences, which contributes to increasing the possible influence of the matrix background on the AM signal. Also, the recovery was found to decrease with increasing sample volumes above 500 μL. If a large sample was used (more than 500 μL), then a fraction of the analyte would break through the pre-column to waste before the entire sample was loaded. Accordingly, the sample volume of the 250 μL serum was selected as a compromise between the required sensitivity and accuracy.

Precolumn switching and column stability

The pre-column switching technique requires a small pre-column, 10 × 4.6 mm i.d., and a six-port switching valve, which was placed between the pre-column and the analytical column. During the injection of a sample, the switching valve was set in the waste position, and the components eluting from the pre-column did not enter the separation column. Just before the component to be determined was eluted, the switching valve was switched to enable it to pass into the analytical column. The pre-column switching valve technique thus prevented proteins from entering the analytical column, and the working life of which was unaffected by the serum injections. The pre-column analytical column combination was, however, affected by the serum injections owing to deterioration of the pre-column. After a certain number of injections, the peak efficiency decreased and the pre-column backpressure increased. The original conditions were restored by replacement of the pre-column. With the chosen phase system, 100 injections of 250 μL of serum could be made before the initial value of the efficiency and column backpressure was changed by 10%, which was a reasonable limit for a change of the pre-column. It was possible to accept a larger change of these parameters; however, under these conditions, standardization was necessary at closer intervals to ensure accurate quantitation. The preparation of such a pre-column was simple and economic and reduced the time and costs of renewal.

Filtration of the serum samples prior to injection is of great importance, as thawed samples often contain clots and solid particles, which on injection will give an immediate increase in the pre-column backpressure owing to restrictions of the inlet filter. Frozen serum samples should first be allowed to thaw to room temperature, followed by filtration through 45-μm filters to avoid obstruction of the protein-coated column inlet filter. This caution was important for the successive analysis of serum samples without pressure trouble. The samples were filtered using 0.45 μm disposable filters (Agilent premium syringe filters with mini-tip regenerated cellulose polypropylene, 13 mm diameter).

Validation

Linearity

The calibration range was established through consideration of the practical range necessary, according to the AM concentration after administration of therapeutic doses of the drug. The standard curve from directly injected spiked serum samples was constructed by plotting the measured peak area against concentration of AM in the concentration range of 12–120 ng/mL. The coefficient for the linear equation $y = a + bC$ was

Table I
Characteristic Parameters for the Regression Equations of the Proposed HPLC Method*

Parameters	AM
Calibration range (ng/mL)	12–120
LOD (ng/mL)	2.60
LOQ (ng/mL)	8.00
Slope (b)	0.9660
Standard error of slope	7.55×10^{-1}
Intercept (a)	1.8939
Standard error of intercept	9.73×10^{-3}
Correlation coefficient (r^2)	0.9995

* $y = a + bC$, where C is the concentration of AM and GP in ng/mL, and y is the peak area.

Table II
Intra-and Inter-day Validation for the Determination of AM Spiked in Drug-Free Human Serum by Direct Injected Technique

Concentration (ng/mL)	Intra-day assay			Inter-day assay		
	Recovery % ± SD*	CV (%)	Mean RE %	Recovery % ± SD*	CV (%)	Mean RE %
AM						
15	92.90 ± 4.08	4.39	−7.10	91.69 ± 4.22	4.60	−8.31
40	95.96 ± 3.24	3.38	−4.04	94.70 ± 3.69	3.90	−5.30
100	97.62 ± 1.95	2.00	−2.38	96.94 ± 2.27	2.34	−3.06

* Mean and SD for the five determinations

calculated using linear regression least square method, where y is the peak area, and C denotes the concentration in ng/mL. Characteristic parameters of the linear calibration curve are shown in Table I.

Assay detection and quantification limits

The limit of detection (LOD), defined as the lowest concentration of AM that can be clearly detected above the base line signal, is estimated as three-times the signal-to-noise ratio. The LOD was determined ($n = 6$) by injection of spiked serum samples with AM in decreasing concentrations. The LOD was found to be 2.60 ng/mL. The LOQ is often defined as 10 times the signal-to-noise ratio. The LOQ was determined ($n = 6$) by injection of the spiked serum samples with AM in decreasing concentrations. The precession was calculated for each concentration. Then, the LOQ was calculated as the concentration, where the precession was less than 20% and was found to be 8.00 ng/mL.

Recovery, precision, and accuracy

The average recoveries (%) of AM spiked in the human serum were 92.90%, 95.96%, and 97.62% (Table II). The reason for the quantitative recovery may be due to the fact that deproteinization is carried out chromatographically. The intra-day CV% ranged from 2.00% to 4.39%, while the inter-day CV% ranged from 2.34% to 4.60% (Table II). At the same concentration levels, the intra-day RE was between −7.10% to −2.38%, while the inter-day RE ranged from −8.31% to −3.06%, which were well within the generally accepted limits for bioanalytical method.

Specificity, selectivity, and applicability

To examine the possible interferences of endogenous components, six serum samples from different volunteers were analyzed individually during method validation. The results did not show any significant chromatographic interference around the retention time of AM. The selectivity of the assay was evaluated by analysis of a group of potentially co-administered drugs with AM, such as paracetamol, caffeine, aspirin, pseudoephedrine, oxomemazine, guaiphenesin, and theophylline. The retention times for these drugs under the chromatographic conditions for the AM assay were determined. The results showed that there was no interference with the retention time of AM, and therefore the present procedure can be applied for its determination in serum. Also, the method described here has been applied to monitor the concentration of AM in human serum after a therapeutic dose of AM. A single 30 mg dose (30 mg per tablet) of AM was administered orally to an adult male volunteer aged 20 years who was advised about the nature and purpose of the study. The volunteer possessed good health and had not taken any medication for at least 2 weeks prior to the study. The preliminary experiments showed that there was no interference with the retention time of AM (Figure 2), and the concentration of AM in volunteers determined by this method agreed with the values obtained from an alternative method (16), making the technique promising for pharmacokinetic studies in humans.

Stability

To ensure the reliability of the results in relation to the handling and storing of AM serum samples, the stability studies were carried out at low and high concentration levels (20 and 100 ng/mL). The long-term stability of AM in human serum was assessed by carrying out the experiment after 4 months of storage at −20°C. Freeze and thaw stability for three cycles was also determined. The samples were obtained over three freeze-thaw cycles by thawing at room temperature and then refreezing at −20°C. The short-term room temperature stability was examined by keeping serum samples at room temperature for 12 h. Twenty-four hour stability of serum sample storage at 5°C was also determined. The concentration of AM after each storage period was related to the initial concentration as determined for the samples that were freshly prepared. Experiments showed that there was no difference in the mean peak areas after one, two, or three freeze-thaw cycles and freshly prepared serum samples. Also, AM, in serum samples exhibited no chromatographic changes for one day when stored refrigerated at 5°C and 4 months at −20°C. Processed serum samples were found to be stable at room temperature upon standing for at least 8 h.

Robustness

Variation of the pH of the mobile phase by ± 0.15 and its organic strength by ± 2% did not have a significant effect on HPLC chromatographic resolution.

Conclusion

Effective on-line extraction procedure using protein-coated μ-Bondapak CN silica pre-column in column-switching HPLC

was investigated for the routine analysis of AM in serum. The method described herein was proven to be simple, sensitive, selective, precise, and accurate for challenging and complex biofluids. Because the recoveries of AM ranged from 94.00% to 97.62%, the internal standard could be eliminated, and this feature was one of the most important advantages over the classical HPLC methods. The concentration of AM in the volunteer determined by this method agreed well with the values obtained from an alternative method (16), making the described HPLC system promising for pharmacokinetic studies in humans.

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