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SOLID-PHASE EXTRACTION OF ACETAZOLAMIDE FROM BIOLOGICAL FLUIDS AND SUBSEQUENT ANALYSIS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A sensitive, relatively fast and simple to operate high-performance liquid chromatographic method for the determination of acetazolamide in plasma and saliva is described. Quantitative extraction of the drug from both plasma and saliva was achieved using commercially available reversed-phase octadecylsilane-bonded silica column (Bond-Elut C_{18}, 2.8 ml capacity). Acetazolamide and the internal standard are retained on the Bond-Elut C_{18} column and reproducibly recovered by elution with methanol. Liquid–liquid partition chromatography, carried out on a 30-cm μPorasil column (10-μm porous silica) using a mobile phase consisting of dichloromethane–ethanol–water–glacial acetic acid (500:65:65:1), provided adequate separation with acceptable retention times. Acetazolamide levels in the region 50–100 ng/ml can be determined in 100 μl of plasma or 200 μl of saliva employing ultraviolet detection at 254 nm with a sensitivity of 0.005 absorbance units full scale. Although the method is primarily used to determine steady-state drug levels in paediatric patients, its general applicability is illustrated by the 24-h plasma and saliva concentration profiles obtained from a male volunteer following oral administration of acetazolamide.

INTRODUCTION

Acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulphonamide) is a carbonic anhydrase inhibitor primarily used to control intraocular pressure in the treatment of glaucoma. However, since the recognition of its anticonvulsant properties in 1952 [1], acetazolamide has also been used with some success in the treatment of epilepsy [2–4].

A variety of approaches have been used to measure the drug in biological fluids. These include an enzymatic method based on the inhibition of carbonic

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anhydrase by acetazolamide [5], a colorimetric assay [6], several high-performance liquid chromatographic (HPLC) techniques [7—10] and one using gas chromatography (GC) [11]. Despite superceding the earlier enzymatic and colorimetric assays, chromatographic methods are generally unsuitable for paediatric applications because they require relatively large volumes of plasma [7, 9]. Furthermore, those methods which comply with the sample size limitations imposed by this patient group usually involve lengthy sample preparation procedures prior to analysis [10, 11]. However, Chambers et al. [9] appear to have overcome the latter problem, although the advantage of improved sample preparation is largely countered by the prolonged retention times involved. This improvement was achieved by back-extracting the drug into alkaline buffer following extraction with ethyl acetate, thus providing a sample suitable for analysis by HPLC using reversed-phase conditions. The determination of acetazolamide in plasma by reversed-phase HPLC, however, is susceptible to interference from theophylline and other dimethylxanthines derived from caffeine, which tend to co-elute with the drug [12—14]. It is unfortunate, therefore, that the aqueous sample extracts obtained using the above method [9] preclude normal-phase chromatography due to incompatability with the predominantly non-polar mobile phase constituents.

Since none of these methods is ideally suited to our needs, we have developed a technique for the determination of acetazolamide in plasma and saliva which overcomes the apparent shortcomings of previous attempts. The method described differs from alternative approaches in two respects. First, it uses reversed-phase octadecylsilane-bonded silica columns to provide a clean extract of the drug for analysis, thus overcoming the problem of tedious sample preparation. Secondly, chromatography is performed on a silica column using a water-saturated mobile phase so that relatively short retention times for acetazolamide and the internal standard can be achieved.

EXPERIMENTAL

Materials

Ethanol (absolute alcohol AR grade) was obtained from James Burrough (London, U.K.). Dichloromethane (general purpose reagent grade), hydrochloric acid, glacial acetic acid (AnalaR quality) were all purchased from BDH (Poole, U.K.). Methanol (HPLC grade) was bought from Rathburn (Walkerburn, U.K.). Acetazolamide and propazolamide were gifts from Lederle Labs. (Gosport, U.K.). Bond-Elut C_{18} columns (2.8 ml capacity) were manufactured by Analytichem International (Harbor City, CA, U.S.A.) and supplied by Jones Chromatography (Llanbradach, U.K.).

Preparation of mobile phase

A 65-ml volume of water (doubly glass-distilled), an equal volume of ethanol and 500 ml of dichloromethane were mixed for 1 h in a stoppered conical flask using a magnetic stirrer. Following separation in a glass separating funnel, the lower organic-rich layer was removed, and its volume determined. After the addition of glacial acetic acid (equivalent to 1% of the measured volume) and final mixing, the solvent was filtered through a 0.5-μm Millipore filter (Type FH) and degassed prior to use.

Chromatography

The high-performance liquid chromatograph consisted of a Waters Assoc. Model 6000A constant-volume pump, a U6K universal loop injector and a Model 440 UV detector. Absorbance was measured at 254 nm with a sensitivity of 0.005 absorbance units full scale. A standard 30 cm × 3.9 mm I.D. stainless-steel μPorasil column (10-μm porous silica) was used in conjunction with a Waters Assoc. Guard-Pak precolumn module containing a Guard-Pak silica cartridge. Chromatography was carried out at ambient temperature with freshly prepared mobile phase using a flow-rate of 2.0 ml/min. This produced a back-pressure in the region of 12.2 MPa (1800 p.s.i.).

Equilibration of analytical column

Prior to use, the μPorasil analytical column was washed with dichloromethane to displace the hexane used for storage purposes. Methanol was then pumped through the column overnight at a flow-rate of 1 ml/min with the solvent recycling. The column was finally equilibrated with mobile phase at 1 ml/min until baseline stability was achieved. At this point the column was considered ready for the analysis of samples and the flow-rate was increased to 2.0 ml/min.

Preparation of internal standard solution

Propazolamide (10 mg) was dissolved in ethanol (100 ml) to give a concentration of 100 μg/ml. The working internal standard concentrations of 4 and 1 μg/ml for plasma and saliva samples, respectively, were achieved by taking 4 and 1 ml of the concentrated propazolamide solution (100 μg/ml) and diluting to 100 ml with water in a volumetric flask. The internal standard was incorporated into the sample as described under *Extraction procedure*.

Extraction procedure

Plasma. Extraction of acetazolamide from plasma was achieved using reversed-phase octadecylsilane-bonded silica columns (Bond-Elut C_{18}, 2.8 ml capacity). These were conditioned prior to use by drawing two column volumes (2 × 2.8 ml) of methanol followed by a similar volume of 0.1 *M* hydrochloric acid through the column under vacuum. On releasing the vacuum, 100 μl of the plasma sample followed by 50 μl of the internal standard solution (propazolamide, 4 μg/ml in water) were loaded onto the column. After standing for 1 min, the sample was drawn through the column by re-applying the vacuum. The vacuum was then released, allowing a short equilibration period (2 min) before proceeding to the washing stage. Washing was facilitated by drawing one column volume (2.8 ml) of 0.1 *M* hydrochloric acid followed by a similar volume of water through the column under vacuum. Acetazolamide and propazolamide were eluted from the column with methanol. This process was accomplished in two stages, the first 200 μl of methanol were discarded (allowed to run to waste) and the drug together with the internal standard were eluted with a further 300 μl of methanol which was collected. This fraction was evaporated to dryness under nitrogen at 55°C and the residue re-constituted in 250 μl of mobile phase. Aliquots (25 μl) were injected into the chromatograph.

Saliva. In order to overcome problems resulting from the high viscosity of saliva, samples were frozen at −20° C, thawed and centrifuged (ca. 600 *g* for 10 min) prior to use, otherwise the extraction of acetazolamide from saliva is essentially the same as that for plasma. Acetazolamide levels in saliva, however, are considerably lower than those found in plasma; consequently a number of modifications were necessary to improve sensitivity. The sample volume was increased to 200 μl of saliva and the internal standard concentration was reduced (50 μl propazolamide, 1 μg/ml in water). In addition, the volume of mobile phase used for reconstitution following sample evaporation was also reduced to 150 μl and the injection volume increased to 50 μl.

Large numbers of samples were conveniently handled using a special Vac-Elut vacuum apparatus (Analytichem International) supplied by Jones Chromatography. This allowed ten cartridges to be used simultaneously, and samples were drawn through the cartridges under vacuum at controllable flow-rates. When sample loading and washing was complete, recovery of the drug was achieved by eluting with the appropriate solvent. Tubes held in a rack within the vacuum apparatus enabled fractions to be collected; on release of the vacuum these tubes were removed for analysis.

Preparation of standards for calibration

Plasma. A stock solution containing 1 mg/ml acetazolamide in ethanol was diluted with water to give additional solutions with concentrations of 100 and 10 μg/ml. Calibration standards were prepared by making appropriate additions of these standard solutions to aliquots of plasma producing concentrations of 125, 250, 500 ng/ml, 1, 5, 10 and 20 μg/ml, respectively. Of these standards, 100 μl were subjected to the previously described procedure for plasma samples. Following extraction, 25-μl aliquots were injected into the chromatograph and the graph of peak-height ratio (acetazolamide/internal standard) versus the actual acetazolamide concentration was plotted.

Saliva. Aliquots of saliva were spiked with appropriate volumes of the stock solutions described above to provide concentrations of 100, 250, 500 ng/ml, 1 and 5 μg/ml, respectively. These saliva standards (200 μl) were subjected to the previously described extraction procedure for saliva samples. Chromatography of 50-μl injections of the extracted samples provided the peak-height ratio data (acetazolamide/internal standard) for the saliva calibration graph.

Extraction recovery experiment

Plasma. Plasma samples were spiked with acetazolamide to give concentrations of 500 ng/ml and 5 μg/ml, respectively. These were subjected to the previously described extraction procedure for plasma and 25 μl of the extract were injected into the chromatograph. Following analysis, peak heights from extracted samples were compared with those obtained from injections of standard solutions and the percentage recovery was calculated.

Saliva. Samples were prepared in saliva by adding appropriate quantities of acetazolamide to give concentrations of 250 ng/ml and 1 μg/ml, respectively. These samples were extracted by the method outlined and 50-μl aliquots were injected into the chromatograph. The peak heights obtained were compared with those from injections of solutions of authentic acetazolamide and the extraction efficiency was determined.

RESULTS

Fig. 1A shows the chromatogram obtained following injection of solutions of authentic acetazolamide and propazolamide (the internal standard) which are eluted from the column after 4.50 and 3.45 min, respectively using the conditions described. A typical chromatogram of extracted blank plasma is illustrated in Fig. 1B whilst Fig. 1C represents the chromatogram obtained from a patient following oral ingestion of acetazolamide (250 mg). By comparing Fig. 1B and C it is clear that blank plasma is free from endogenous components which may interfere with the quantitation of acetazolamide. The chromatograms of extracted saliva are shown in Fig. 2A and B. The former represents extracted blank saliva from a patient prior to taking acetazolamide whilst the latter illustrates saliva obtained from the same patient several hours later.

Extraction of acetazolamide from plasma using Bond-Elut C_{18} extraction columns provided recoveries of 91.4 and 96.6% for plasma concentrations of 500 ng/ml and 5 μg/ml, respectively (see Table I). The corresponding coefficients of variation were 1.27 and 2.43% for five determinations in each

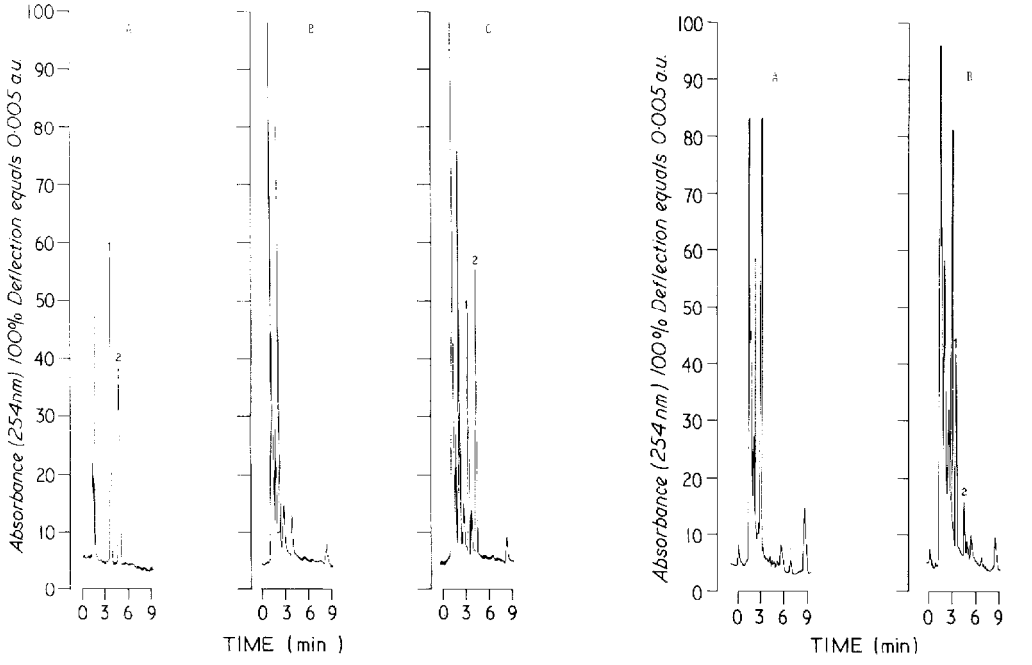

Fig. 1. (A) Chromatogram of authentic components prepared in mobile phase prior to injection. (B) Chromatogram of extracted blank plasma. (C) Chromatogram of extracted plasma obtained from a patient following oral ingestion of acetazolamide. The internal standard (1) and acetazolamide (2) peaks are adequately separated from endogenous components. Following analysis this sample was found to contain 2.92 μg/ml acetazolamide. Peaks: 1 = propazolamide (internal standard); 2 = acetazolamide.

Fig. 2 (A) Chromatogram of extracted blank saliva. (B) Chromatogram of extracted saliva from a patient several hours after taking acetazolamide orally. The concentration of acetazolamide measured in this saliva sample was 98.5 ng/ml. Peaks: 1 = propazolamide; 2 = acetazolamide.

TABLE I

EXTRACTION RECOVERY OF ACETAZOLAMIDE AND PROPAZOLAMIDE FROM PLASMA AND SALIVA

Sample	Component	Concentration (μg/ml)	n	Recovery (%)	Coefficient of variation (%)
Plasma	Acetazolamide	0.50	5	91.4	1.27
	Acetazolamide	5.00	5	96.6	2.43
	Propazolamide	2.00	20	96.3	2.54
Saliva	Acetazolamide	0.25	5	96.4	3.49
	Acetazolamide	1.00	5	89.1	2.40
	Propazolamide	0.25	10	87.7	1.89

case. The internal standard, propazolamide, was also extracted quantitatively from plasma using this approach. The recovery value of 96.3% with a coefficient of variation equal to 2.54% was obtained at the working concentration of 2 μg/ml for twenty measurements. Table I also shows that acetazolamide and propazolamide are extracted quantitatively from saliva using Bond-Elut C_{18} columns. Recovery values and coefficients of variation are 96.4 and 3.49%, and 89.1 and 2.4% for saliva acetazolamide concentrations of 250 ng/ml and 1 μg/ml, respectively (n = 5 at both concentrations). The recovery of propazolamide at the working internal standard concentration of 250 ng/ml was 87.7% with a coefficient of variation equal to 1.89% (n = 10). It appears, therefore, that the extraction efficiency and reproducibility are independent of the concentration of acetazolamide at the levels investigated in both plasma and saliva samples. Furthermore, the values obtained for propazolamide are of the same order.

Calibration curves were obtained by comparing the peak-height ratio (acetazolamide/internal standard) with the actual concentration of acetazolamide in spiked aliquots of plasma and saliva. In both cases, the relationship was linear over the calibration range (0—20 and 0–5 μg/ml for plasma and saliva, respectively). The correlation coefficients (r) and slope values were 1.00 and 0.39 (n = 36) and 1.00 and 3.34 (n = 22) for plasma and saliva, respectively.

The effects of sample storage on reproducibility of results were also examined. In the case of plasma, analyses were carried out in duplicate, at weekly intervals, on replicate samples containing either 500 ng/ml or 5 μg/ml acetazolamide following storage at 0—4°C for a period of four weeks maximum. The mean recovery and coefficients of variation (see Table II) were 100.6 and 6.43%, and 99.68 and 2.6% for the low and high plasma concentrations, respectively. The extraction efficiency and reproducibility of the assay are apparently unaffected by storage at temperatures between 0 and 4°C for at least four weeks. For saliva, two series of samples containing 250 ng/ml and 1 μg/ml acetazolamide, respectively, were stored at −20°C and assayed at weekly intervals for one month, followed by monthly intervals for four months. The mean recovery and coefficients of variation, as reported in Table II, were 100.4 and 4.68%, and 99.5 and 3.9% for the low and high concentrations, respectively. Storage of saliva samples at −20°C for up to four months, there-

TABLE II

EFFECTS OF STORAGE ON THE DETERMINATION OF ACETAZOLAMIDE IN PLASMA AND SALIVA

Plasma samples were stored at 4° C and assayed at weekly intervals over a period of four weeks; saliva samples were stored at −20° C and assayed at weekly intervals for one month followed by monthly intervals for four months.

Sample	Concentration (μg/ml)		Recovery (%)	Coefficient of variation (%)	*n*
	Added	Determined			
Plasma	0.50	0.51	100.60	6.43	10
	5.00	4.98	99.68	2.60	10
Saliva	0.25	0.251	100.40	4.68	13
	1.00	0.995	99.50	3.90	13

TABLE III

CONCENTRATIONS OF ACETAZOLAMIDE IN PLASMA AND SALIVA FOLLOWING ORAL ADMINISTRATION TO AN ADULT MALE VOLUNTEER

Time (h)	Concentration (μg/ml)		$\frac{\text{Saliva concentration}}{\text{Plasma concentration}} \times 100$ (%)
	Plasma	Saliva	
0	0	0	–
0.5	6.16	0.17	2.8*
1	14.60	0.26	1.8
2	8.70	0.19	2.2
3	6.10	0.10	1.6
4	5.54	0.09	1.6
6	4.96	0.08	1.6
8	2.92	0.06	2.1
13	1.98	0.04	2.0
24	0.62	<0.02	–

*Result excluded since anomalous saliva values may ocur if sampled within 1 h of oral administration [11].

fore, appears to have a minimal effect on the recovery and reproducibility of the assay described.

The general applicability of the method is illustrated by the 24-h plasma and saliva concentration profiles, listed in Table III. These were obtained following oral administration of 250 mg of acetazolamide to an adult male volunteer. Although the saliva concentrations are much lower than plasma values, the 24-h concentration profiles follow similar patterns. This is more obvious if the results are represented graphically in the form of a semilogarithmic plot of concentration versus time, as illustrated in Fig. 3. From these graphs, the half-lives ($t_{½}$) of the biexponential decay curves can be calculated. In the case of plasma, determined $t_{½}$ values for the initial and terminal elimination phases are 1.59 and 6.3 h, respectively, whilst in saliva the respective values are 1.45 and 7.45 h.

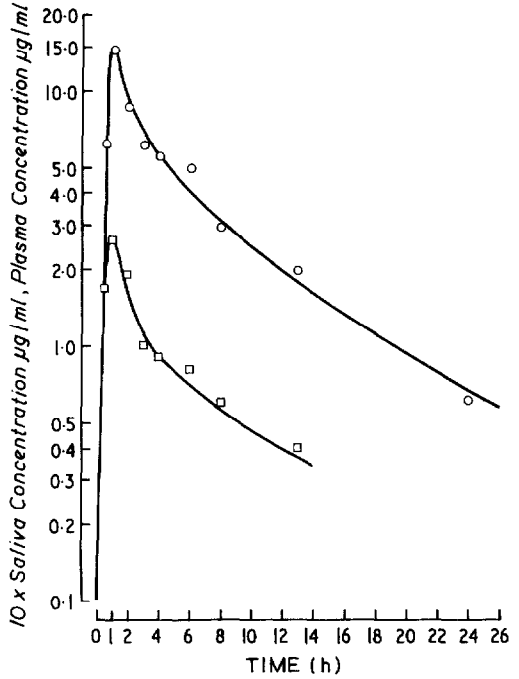

Fig. 3. Semilogarithmic plot of plasma and saliva acetazolamide concentrations versus time: (□) 10 × saliva concentration; (○) plasma concentration.

DISCUSSION

The method outlined has been developed to monitor steady-state levels of acetazolamide in the relatively small samples usually obtainable from young children. However, for general use and for applications involving pharmacokinetic studies in particular, the method is sufficiently sensitive to allow levels in the region 50–100 ng/ml to be measured in only 100 μl of plasma or 200 μl of saliva. In addition to permitting the analysis of acetazolamide in saliva, the advantage of this method compared with alternative HPLC procedures [7–10] is that, in most cases, overall sensitivity is maintained but with an appreciable reduction in sample size. Despite a large sample requirement for salivary measurements, the GC method of Wallace et al. [11] is undoubtedly the most sensitive, although sample preparation is tedious and involves derivatisation prior to analysis. By comparison, the introduction of Bond-Elut C_{18} cartridges to provide clean samples for analysis by HPLC keeps sample handling to a minimum.

The use of solid-phase extraction columns (Bond-Elut C_{18}) provides recovery values in excess of 89% for acetazolamide and greater than 87% for the internal standard with either plasma or saliva (see Table I). These values are comparable with recoveries of 95% in the case of Chambers' group [9], 83.5 ± 6.5% reported by Gal et al. [10], 94% obtained by Hossie et al. [8] and 68–94% using the method of Bayne et al. [7] which were all achieved employing liquid–liquid extraction techniques.

The choice of chromatographic conditions used in this assay has been

influenced by two important factors. First, reports of acetazolamide co-eluting with theophylline [12, 13] and theobromine [14] in HPLC assays employing reversed-phase conditions indicate the possibility of interference from either dietary sources or medicaments. Secondly, we considered other projects currently under investigation in this laboratory including the use of a silica column with a water-saturated mobile phase which we have previously evaluated for the determination of corticosteroids in biological fluids [15]. Considering, therefore, the possibility of interference using reversed-phase conditions [12—14], we decided to evaluate this alternative approach [15] in order to establish a common chromatographic system for monitoring a variety of drugs. To elute acetazolamide and propazolamide from the μPorasil column with suitable retention times, however, modification of the original mobile phase was necessary. The retention times of 4.50 and 3.45 min for the drug and the internal standard, respectively, are considerably faster than those reported by Chambers' group [9], marginally faster than those of Bayne et al. [7] and of Gal et al. [10], and comparable with the values obtained by Hossie et al. [8].

Since caffeine and its N-demethylated metabolites are usually present in plasma, owing to widespread consumption of caffeine-containing beverages, they were examined for potential interference with the quantitation of acetazolamide using this method. Caffeine and its metabolites are extracted from plasma using the procedure described but they are eluted from the analytical column rapidly (retention times < 3 min) and so do not present any problems regarding the quantitation of acetazolamide. In addition, a variety of drugs were also evaluated for interference with the assay. Chlorothiazide, carbamazepine, phenobarbitone, salicylic acid, acetylsalicylic acid, benzylpenicillin, netilmicin, ampicillin, cefuroxime, cortisol, phenytoin and diazepam did not interfere. Prednisolone and acetaminophen had retention times very similar to that of propazolamide. However, there has been no evidence of interference in extracted plasma and saliva samples to date; the chromatograms of blank plasma and saliva shown in Figs. 1B and 2A are typical of many such samples analysed.

The storage stability trials indicate that plasma containing acetazolamide can be stored at approx. 0—4°C in the refrigerator for up to four weeks without significant change (see Table II). This is at variance with the findings of Gal et al. [10] who reported that 17% loss of acetazolamide occurred within one week when plasma samples were stored at between 2 and 4°C. To prevent this, they suggested storage at —20°C. However, although no significant losses in acetazolamide were oberved in this study following storage at 0–4°C, there was some deterioration in the consistency of the extracted plasma blanks. Small extraneous peaks which were occasionally observed in the chromatogram in close proximity to both the acetazolamide and internal standard peaks, presented the possibility of problems with accurate quantitation. As there is no advantage to storing plasma at 0—4°C, and since it is essential to deep-freeze (—20°C) and thaw saliva in order to reduce the high viscosity of fresh saliva and ensure a reasonable flow-rate through the extraction column, both plasma and saliva samples were kept at —20°C until analysed. Upon adopting this practice, the consistency of the chromatograms from extracted blank plasma

was maintained. According to Gal et al. [10] plasma samples are stable under these conditions for at least eight weeks. In the case of saliva, Table II clearly shows that quantitative recovery was maintained throughout (> 99.5%) with excellent reproducibility (coefficient of variation < 5%) when samples were stored at −20° C for up to four months.

The 24-h plasma concentration profile obtained from a male volunteer following a 250-mg oral dose of acetazolamide (see Table III) shows a peak concentration of 14.6 μg/ml after 1 h. This is in excellent agreement with published data from similar studies [7, 8, 10, 11]. Although saliva concentrations were only approx. 2% of plasma values, they reflected plasma levels closely throughout the study with the maximum of 160 ng/ml also occurring after 1 h. The saliva/plasma ratio, expressed as a percentage, remained constant with both time and acetazolamide concentration with one exception (the 0.5-h sample). However, Wallace et al. [11] noted a tendency for saliva to produce anomalous results if sampled within 1 h of oral administration of the drug; consequently we have excluded our first saliva sample result in calculating the mean saliva/plasma ratio. The mean value (± standard error of the mean) of 1.84 ± 0.09% is approximately twice the figure obtained by Wallace et al. [11] using GC. The relationship between saliva and plasma acetazolamide was examined further using linear regression analysis. The correlation coefficient (r) was found to be 0.985 and the slope was 0.018 (n = 8), thus confirming a strong correlation between saliva and plasma concentrations.

The elimination of acetazolamide from plasma follows a similar biphasic decay curve to that previously described by Hossie et al. [8] and by Wallace et al. [11]. Furthermore, the saliva level decreased at comparable rates. Plasma $t_{½}$ values are 1.59 and 6.3 h compared with saliva $t_{½}$ values of 1.45 and 7.45 h for the initial and final elimination phases, respectively. These figures compare favourably with previously published plasma $t_{½}$ values of 95 min for the initial phase [16, 17] and 4.1 h [18], 5 h [19] and 8 h [20, 21] for the final phase.

The excellent agreement between results obtained using the method described and those reported in earlier papers [7, 8, 10, 11] is a clear indication that this method is a valid alternative.

Clinical results from paediatric patients will be published later.

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