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Determination of taurine in biological samples and isolated hepatocytes by high-performance liquid chromatography with fluorimetric detection

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Abstract

A high-performance liquid chromatographic method with fluorimetric detection is described for the routine and selective determination of taurine in urine, serum, tissues and isolated hepatocytes. The preparation and use of ion-exchange resins to extract taurine from biological samples is included. Taurine was derivatised with *o*-phthalaldehyde/2-mercaptoethanol prior to injection onto a C_{18} column (LiChrospher^R 100 RP-18, 5 μm, 125 × 4 mm I.D.). Isocratic elution of the adduct was carried out using NaH_2PO_4 (0.05 *M*, pH 5.4) in methanol and water (43:57, v/v). Homoserine was used as an internal standard to facilitate the standardisation and quantitation of samples and analysis was completed in 6 min with homoserine and taurine eluting after 3 and 4 min, respectively. The method will detect 0.5 pmol of taurine on the column. Appropriate dilutions of these biological samples enable these samples to be assayed on an autosampler, using the same standard curve. Concentrations of taurine in human, dog and rat urine, rat liver, serum and isolated hepatocytes are reported.

1. Introduction

Taurine is present in biological fluids and tissues in high concentrations (1–65 m*M*) as a non-protein, free β-amino acid. The function of such high levels of taurine in a variety of tissues is the subject of much research. It has been demonstrated that taurine is able to act as a neurotransmitter [1], antioxidant [2], modulator of intracellular calcium levels [3], and osmolyte [4]. Taurine conjugates with bile acids [5], xenobiotics [6], and forms the relatively stable taurochloramine with hypochlorous acid. Taurine is considered to be a major end-product of sulphur amino acid metabolism [7] which may be excreted in the urine.

We have proposed that levels of urinary taurine may provide a useful non-invasive marker of hepatotoxicity and perturbations in sulphur amino acid and protein metabolism as these conditions alter urinary and liver concentrations of taurine [8–10]. This work has required the rapid measurement of taurine in a variety of tissues including urine and isolated hepatocytes. Many methods for measuring taurine have been published including gas chromatography of the N-pentaflurobenzoyl di-*n*-butylamide derivative of taurine [11], HPLC analysis of 1-naphthylisocyanate derivatives of amino acids, including taurine [12] and the HPLC separation of taurine followed by post-column fluorescence reaction with thiamine [13].

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Recent developments in assays for taurine and its metabolic precursors and their applications are discussed in a review by Shihabi *et al.* [14]. We have adapted the straightforward method of Larson *et al.* [15] with some similar modifications to those made by Quilligan *et al.* [16] to accomodate larger tissue volumes. This method utilises an initial clean-up procedure for samples using dual-bed Dowex resins [17,18] to remove all interfering amino acids and one of the most sensitive and commonly used derivatisation techniques (*o*-phthalaldehyde/mercaptoethanol) to produce a fluorescent adduct [14–17,19–21]. The isocratic elution of this adduct by reversed-phase HPLC enables as little as 0.5 pmol taurine on the column to be determined by fluorimetric detection (*i.e.* in hepatocyte preparations). Quantification is made easier by the incorporation of an internal standard, homoserine, in the samples [22]. When the concentrations of taurine are higher (1 nmol or more on the column, *e.g.* in rat urine samples) it is possible to use UV absorbance detection at 350 nm [23]. The HPLC separation of 5–10-μl samples was carried out using a C_{18} column fitted with a guard column. During the course of our routine investigations urinary taurine has been measured in rat, human and dog urine samples as well as in rat liver and isolated rat hepatocytes. Control values are presented here to illustrate the variability in the concentrations of this amino acid in untreated subjects.

2. Experimental

2.1. Chemicals

Methanol (HPLC grade) was purchased from Rathburn (Wakeburn, UK). The following compounds were obtained from Sigma (Dorset, UK): taurine (cell culture tested), homoserine, Dowex resins, *o*-phthalaldehyde (OPA, HPLC grade). Sulphosalicylic acid, boric acid, sodium hydroxide (Aristar) and mercaptoethanol were obtained from BDH (Luterworth, Leicestershire, UK). Creatinine was measured in urine samples using an enzyme assay kit obtained from Boehringer (London, East Sussex, UK). All water was of HPLC grade produced using an Elgastat UHQ water purifier.

2.2. Instrumentation

The chromatographic system used routinely consisted of a Beckman Autosampler 507 table (High Whycombe, UK) fitted with a cooling tray, a Reagent Addition Cassette and a C_{18} column (LiChrospher^R 100 RP-18, 5 μm, 125 × 4 mm I.D.) fitted with a LiChrospher precolumn (4 × 4 mm I.D.; E. Merck, Darmstadt, Germany) [24–26]. This was interfaced with System Gold software, a single Beckman 110A pump and Beckman 157 fluorescence detector fitted with an excitation filter, 305–395 nm and emission filter, 420–650 nm or Waters 484 Tunable Absorbance Detector.

2.3. Preparation of ion-exchange columns for sample clean-up resins

Two resins were used for the extraction of taurine from biological material; they were washed with 3 volumes of UHQ water to remove fines; 100 g of resin were subsequently treated in the following way: (a) Dowex-1-X4 (anion-exchange, in Cl^- form) 100–200 mesh–washed with 250 ml 1 *M* HCl then water until pH was above 2.5; (b) Dowex-50W-X8 (cation-exchange, in H^+ form) 100–200 mesh–washed with HCl (500 ml, 4 *M*, in three washings) then 1 *M* HCl (250 ml) [18,27].

2.4. Columns

Dual-bed columns were prepared by layering 0.5 ml Dowex-1-X4 resin directly onto 1.5 ml Dowex-50W-X8 resin in glass Pasteur pipettes with glass wool in the tip. A plastic reservoir was fitted to hold 4 ml of liquid above the resin. The resins were washed with water (12 ml) before use and regenerated after use with HCl (12 ml, 1 *M*) [28] and stored in tall beakers containing 1 *M* HCl [29]. The method enables the re-use of Dowex packed columns for the initial clean-up of many samples. These columns were used to

process up to 200 μl of sample. If the sensitivity of the detector requires larger initial volumes of the biological material, the two resins can be packed into separate columns (2 ml) and stacked one above the other (Cl^- resin above H^+ resin). In this case the regeneration of the Dowex-50W-H^+ can be improved after extraction from urine samples by passing 10 ml NaOH (1 *M*, Aristar CO_3^{2-} free) before the addition of 12 ml HCl (1 *M*) to both columns.

2.5. Sample preparation

One of the difficulties of measuring taurine in biological samples is the variability in the concentrations which are found, particularly in urine and isolated hepatocytes. The following procedures are based on the analyses of rat urine, liver and serum from male and female rats (Sprague–Dawley, outbred stock, Glaxo Research and Development, 150–350 g) fed a powdered rat and mouse maintenance diet (691 diet, Quest Nutrition, Wingham, Kent, UK), human and dog urine samples and hepatocytes isolated from male Sprague–Dawley rats by the method of Moldeus *et al.* [30].

Urine

All 24-h rat urine samples were collected over ice from animals housed in individual metabolism cages, diluted to 25 ml with UHQ water and centrifuged (2500 *g*, 4°C, 10 min) to remove hair and food debris. The urine was stored (−20° or −80°C in 5-ml aliquots) for future analysis. A further dilution was made (1 + 9) prior to analysis if samples were known to contain >1 μmol ml^{-1} taurine. Human samples were used undiluted. An aliquot of diluted rat urine or undiluted human urine (25–100 μl) was placed onto previously prepared dual-bed ion-exchange resins (see above). The first 25–100 μl of displaced water was discarded.

Liver

Sections of frozen tissue (−80°C) were weighed (0.35–0.45 g) into sulphosalicylic acid (4 ml, 0.2 *M*, 4°C) to precipitate protein and homogenized using a Polytron tissue homogenizer [18,31]. The homogenate was centrifuged (2500 *g*, 10 min, 4°C) and 25-μl aliquots of supernatant were extracted for taurine on dual-bed ion-exchange columns. The use of sulphosalicylic acid has been shown to result in a higher % recovery of taurine from tissue homogenates than perchloric acid or trichloroacetic acid [25].

Serum

Serum was obtained from rats under anaesthesia from the abdominal aorta and put into microtainers (Beckton Dickinson, Rutherford, NJ, USA, for the separation of serum), centrifuged (13 000 *g*, 60 s, MSE microfuge) and stored (−80°C). After thawing at room temperature a 100-μl sample of serum was removed, deproteinated with sulphosalicylic acid (100 μl, 0.2 *M*, 4°C, 5 min) [32] and centrifuged to remove the precipitated protein (11 500 *g*, 2 min, 4°C). An aliquot (50 μl) of the supernatant was placed onto a dual-bed Dowex ion-exchange column and the first 50 μl of eluate were discarded.

2.6. Elution of taurine from the columns

The elution of taurine from the columns was completed using 4.0 ml of UHQ water in 0.5-ml aliquots (Fig. 1). Homoserine (100 μl, 100 μ*M*) was added to the resulting 4 ml of eluate as internal standard [22] to give a final concentration of 2.5 nmol ml^{-1} eluate or 10 nmol/4 ml eluted sample.

2.7. Isolated hepatocytes

Taurine was measured in isolated hepatocyte suspensions ($1–1.5 \cdot 10^6$ cells/ml). Cell suspensions were centrifuged (10 s at 3000 *g*) and an aliquot of medium (100 μl) was placed onto a dual-bed ion-exchange column. A sample (0.5 ml) of suspended hepatocytes was added to sulphosalicylic acid (0.5 ml, 0.2 *M*, 4°C) and precipitated protein removed (11 500 *g*, 2 min, 4°C). Supernatant (200 μl) was added to a dual-bed ion-exchange column. Taurine was eluted

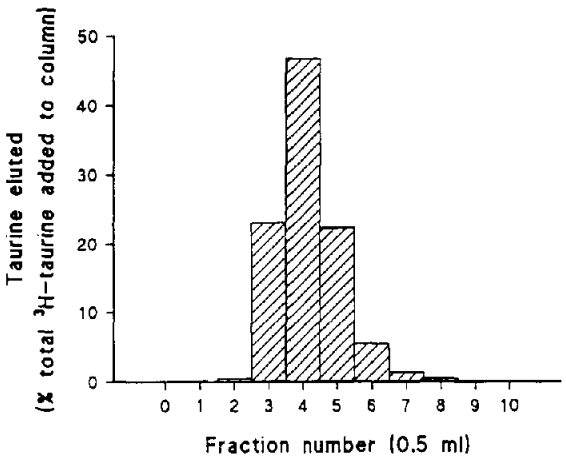

Fig. 1. Elution of taurine from stacked Dowex packed columns showing the % taurine eluted from columns using 0.5-ml aliquots of UHQ water.

from the columns (8 × 0.5 ml UHQ water) and homoserine added as internal standard (40 μl, 100 μ*M* to give 4 nmol/4 ml). Cellular taurine concentration was found by subtraction of the taurine present in the medium.

2.8. Taurine derivatisation and adduct measurement

Taurine was measured in the eluates from ion-exchange columns as a fluorescent adduct formed using *o*-phthalaldehyde (OPA, 40 mg, 0.8 ml absolute ethanol) and mercaptoethanol (40 μl) in 0.5 *M* borate buffer (10 ml, 3.1 g boric acid in 90 ml UHQ water adjusted to pH 10.4 with 5 *M* Aristar NaOH and made up to 100 ml) [15,16,20,21,24,25]. The derivatising solution was diluted 50:50 with UHQ water, filtered through a 0.45-μm filter before use and then stored in a dark vial at room temperature during use. The solution was stable for at least 24 h. The taurine samples eluted from the Dowex resins were acidic. The high pH and molarity of the derivatising solution raised the pH of the reaction mixture above 8 to enable the reaction to proceed.

Taurine in the samples was derivatized by mixing equal volumes of the derivatizing reagent and eluate for 1.5 min before the injection of a sample onto the column (5–10 μl). The fluorescence of the adduct decays with time and therefore the timing of the injection was kept constant [15].

The adduct was chromatographed using isocratic elution (2 ml min^{-1}) with phosphate buffer prepared essentially according to Larson *et al.* [15], then filtered and degassed using a Millipore vacuum filter (0.22-μm CV filter). A flow-rate of 2 ml min^{-1} was used (ambient temperature).

The concentration of taurine in the sample was determined by comparison of the integrated peak area with that of the internal standard (homoserine) using a calibration curve in the range 0.5–40 nmol taurine/4 ml with 4 or 10 nmol homoserine added as internal standard as appropriate for the sample being measured. Results were expressed as μmol kg^{-1} 24 h^{-1} (urinary taurine) in rats and humans or nmol taurine/μmol creatinine in humans, μmol g^{-1} wet weight liver or total μmol/liver, μmol l^{-1} (serum) and nmol 10^6 cells or ml $^{-1}$ medium (hepatocytes).

3. Results

The conditions adopted for the sample preparation, derivatisation and chromatography allowed for the rapid determination of taurine with a sensitivity of 0.5 pmol on the column using fluorimetric detection (100 pmol using spectrophotometric detection). Fig. 1 shows the elution profile of ^{3}H-taurine collected in 0.5-ml fractions from dual-bed ion-exchange columns. As a result of this analysis the taurine samples were eluted from the Dowex resins with 4 ml of UHQ water, derivatised and chromatographed without further processing.

There was a linear relationship between the relative peak area and the amount of taurine in the concentration range of 0.05–1600 nmol ml^{-1} with a correlation coefficient of 0.994–0.999. However, concentrations of standards were usually in the range of 0.05–10 nmol ml^{-1} taurine (0.5 to 50 pmol per injection) to enable a single attenuation level to be used on the detector.

Samples were diluted accordingly. The coefficient of variation, determined from 10 duplicates of the same sample was 2% and the inter-batch variation measured on three separate occasions was 5.5%. The inter-batch variation was found to vary with the life of the column as the retention time was reduced and peaks became less well defined. Fig. 2 shows a typical chromatograph of taurine extracted from rat liver and urine.

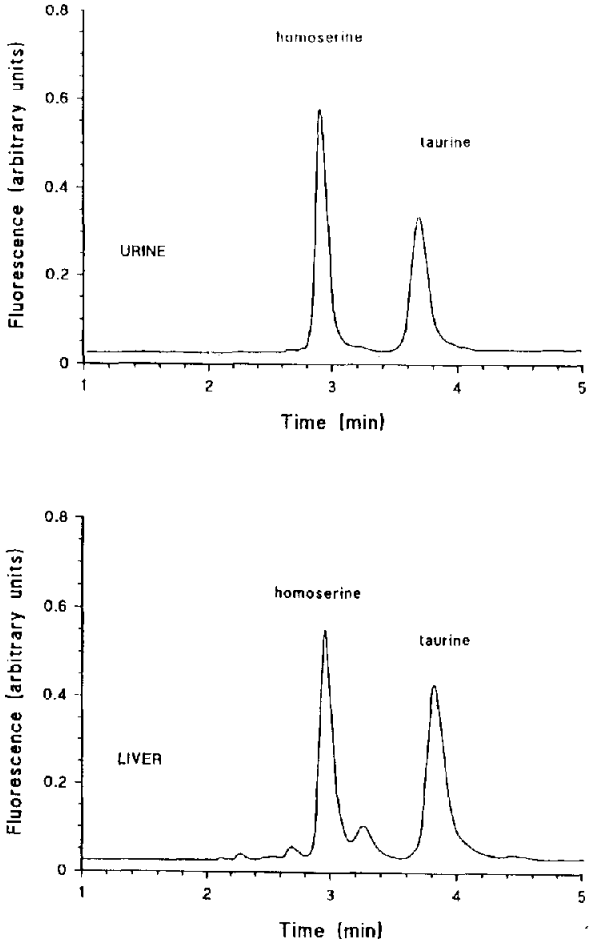

Fig. 2. Typical chromatograms obtained for taurine extracted from rat liver and urine, with homoserine as internal standard. Injection of 5 μl extract from liver and urine containing 2.5 nmol ml^{-1} (12.5 nmol on-column) homoserine and 5 μl derivatising solution. Flow-rate 2.0 ml min^{-1}, ambient temperature.

3.1. Recovery experiments

Recovery experiments were carried out by adding 1 ml of standard amounts of taurine to UHQ water (3 ml + 1 ml 0.2 m*M* homoserine as internal standard) and chromatographing the samples. The same amounts of taurine were added to rat urine, extracted on stacked Dowex resins and eluted with 4 ml UHQ water. Homoserine (1 ml, 0.2 m*M*) was then added as internal standard to the eluates, which were then chromatographed. After subtracting the taurine present in the rat urine (Table 1). The recovery of liver taurine was measured in a similar way using the addition of standards to a 1 + 4 liver homogenate instead of urine. The mean % recovery from liver was 94.73 ± 2.43 (S.D.).

3.2. Urinary taurine

Rats

Levels of taurine in rat urine are high compared with levels in human urine (range 0.5–10 μmol ml^{-1} in rats *vs.* 0.02–1.0 μmol ml^{-1} in humans). Fig. 3 shows urinary levels of taurine in 24-h urine samples collected from different groups of rats (male Sprague–Dawley, 200–350 g). The difference observed between different control groups of rats appeared to be a reflection of the free taurine content in the diet, which varied between batches (range 0.032–2.0 μmol g^{-1} food). Thus, groups a and d were maintained on a diet containing 0.032 μmol taurine/g food and groups b, c and e were maintained on diets containing 2.0 μmol taurine/g food.

Human

Taurine was measured in spot urine collections made from volunteers (12 male and 11 female) and over 2–3 days (Fig. 4). The variation in the excretion of urinary taurine between individuals was thought to be partially diet related. Complete 24-h urine collections were also made. Urinary taurine were expressed as a ratio with urinary creatinine (nmol taurine/μmol creatinine) as well as absolute values.

Mean taurine excretion was slightly lower in

Table 1
Recovery of taurine from rat urine

Concentration of taurine added to sample (m*M*)	Mean Tau:Hse of standards	Mean Tau:Hse standards extracted from rat urine	Recovery (%)
0.4	2.50	2.47	98.9
0.35	2.07	1.94	94.1
0.3	1.79	1.76	98.3
0.25	1.42	1.44	101.3
0.2	0.83	0.83	100.0
0.15	0.59	0.61	103.5
0.1	0.39	0.38	96.6
0.05	0.17	0.17	100.0

Standard amounts of taurine were measured in urine extracts. Values represent the recovery of standards after the subtraction of taurine present in the urine at the start. Values are the mean peak-area ratios of taurine (Tau):homoserine (Hse) ($n = 2$). Mean % recovery 99.59% ± 2.13 (S.D.).

the female volunteers than the male volunteers, when expressed as an absolute value but higher as creatinine corrected values. There was also greater variation in this group (Table 2).

Dog

Urine samples were taken from untreated dogs housed in individual metabolism cages for 16 h (Beagles, male and female, 7.2–12.5 kg). Animals were provided with food *ad libitum* containing 1.05 μmol g^{-1} taurine until they were caged, then food was withheld. Urinary taurine levels are shown in Table 2.

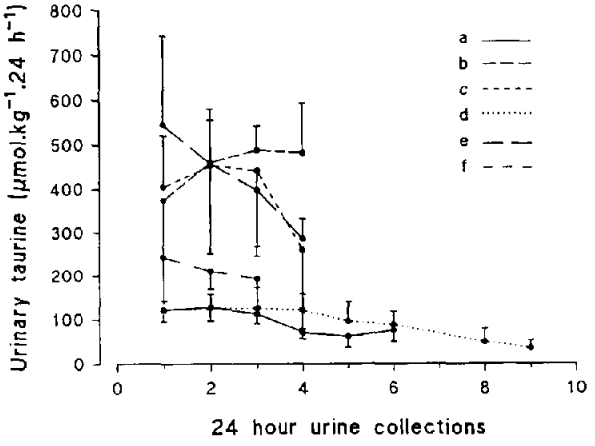

Fig. 3. Urinary taurine levels in six different groups of control rats (a–f) taken from six different experiments over a two-year period. Values are means ± S.D., $n = 4$.

3.3. Liver taurine levels in rats

Liver levels of taurine were measured in the larger right lobe of control groups of male Sprague–Dawley rats (Table 3) at different times to show variability ($n = 4$ in each group). This data was extrapolated to show total liver levels of taurine. In a separate experiment designed to show whether liver levels of taurine could be raised by giving rats taurine in the drinking water, liver lobes were analysed separately for taurine in both treated and control rats (Table 4). The level of taurine in all lobes from untreated rats was low in this study. There was some variation in taurine content between the different lobes of the liver, although none of the values were significantly different. Taurine levels were significantly raised in all liver lobes by administering taurine. The inclusion of taurine in the drinking water did not affect the rats' intake of water.

3.4. Isolated hepatocytes

Isolated hepatocytes show more variation in their taurine content than the livers from which they are isolated. In some preparations more

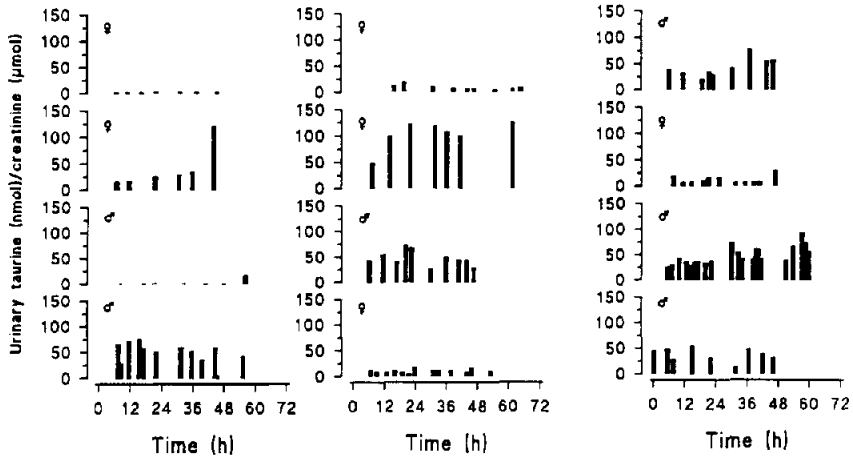

Fig. 4. Urinary levels of taurine in spot urine samples taken from representative male and female volunteers for 48–72 h. Values are nmol taurine/μmol creatinine.

Table 2
Urinary taurine (mean ± S.D.) in 24-h urine collections from male and female human volunteers and in overnight urine samples from control dogs

	Taurine	Taurine/creatinine ratio
Human (female) ($n = 11$)	534 ± 671 μmol/24 h	59.2 ± 59.8
Human (male) ($n = 12$)	601 ± 249 μmol/24 h	35.7 ± 24.6
Dog (female) ($n = 6$)	1.79 ± 1.48 μmol/ml urine	393 ± 210
Dog (male) ($n = 6$)	2.64 ± 1.38 μmol/ml urine	470 ± 197

Values are expressed as total taurine excreted in 24 h (human) or μmol/ml urine (dogs) and as nmol taurine:μmol creatinine.

Table 3
Variation of liver taurine levels in groups of rats measured in different studies (a–g).

Animal group	Liver taurine μmol g^{-1} (wet weight)	Total liver taurine (μmol)
a	5.98 ± 1.24	63.3 ± 12.6
b	4.01 ± 1.26	41.98 ± 11.8
c	7.40 ± 1.08	85.8 ± 12.7
d	11.18 ± 1.76	153 ± 29.0
e	5.12 ± 1.32	66.6 ± 29.8
f	6.16 ± 0.76	73.7 ± 7.7
g	8.95 ± 1.12	122.1 ± 17.2

Values are means ± S.D.; $n = 4$ rats in each group.

Table 4
Concentration of taurine in different lobes of the liver from control rats and rats provided with 3% taurine (w/v) in the drinking water for 4 days

Liver lobe	Concentration of taurine (μmol g^{-1} wet weight)	
	− taurine	+ taurine
1	1.89 ± 0.32	15.80 ± 3.80 [a]
2	2.69 ± 0.84	15.29 ± 4.36 [a]
3	2.18 ± 4.94	14.53 ± 3.78 [a]
4	2.47 ± 0.68	15.57 ± 2.20 [a]
5	4.06 ± 1.84	17.62 ± 5.44 [a]

[a] $p < 0.001$ different from liver lobe from untreated rats, Students t test. Nomenclature used for rat liver lobes: 1 (larger) and 4 (2 small) = right lobes; 2 = left lobe; 3 = median lobe; 5 = posterior or caudate lobe. Values are means ± S.D.; $n = 4$ rats.

than 50% of the taurine in a suspension of isolated hepatocytes was found in the incubation buffer. Values in the range of 1–130 nmol ml^{-1} taurine of buffer incubating 10^6 cells [mean 21.5 ± 32.6 (S.D.) nmol ml^{-1}] were found after 45 min pre-incubation. Cellular taurine also showed considerable variation: range 1–118 nmol taurine/10^6 cells [mean 28.9 ± 34.4 (S.D.)].

4. Discussion

The method reported here brings together many elements from other previously used methods to extract and measure taurine. The inclusion of homoserine in urinary and tissue extracts enables the accurate quantitation of taurine after chromatography. The use of borate buffer at a high pH and osmolarity (10.4, 0.5 *M*) in the OPA solution enables acidic extracts to be derivatised directly without further pH adjustment. Although Porter *et al.* [20] were able to detect 0.1 pmol of taurine, quantitation was difficult. Using this method 0.5 pmol on the column can be measured, therefore, taurine in hepatocytes containing 1 nmol 10^6 cells can be measured without using a lyophilisation step. The chromatographic run time of 5 min is comparable with the lowest reported times [11,15] and the use of a guard column and small volume injections extend the life of the column indefinitely, even with routine use.

The preparation of Dowex resins for sample clean-up and appropriate dilutions of samples and ranges of taurine levels are included here to allow the routine use of the method to be easily adopted. The simplicity and speed of analysis resulting from these improvements has enabled changes in taurine levels to be used as an indicator of liver damage/dysfunction in rats.

The data presented is representative of the variability of levels routinely found in rat and man. Although there was variation in taurine excretion between groups of rats in different studies this has not precluded the use of taurine as a marker of liver dysfunction [9,10], as adequate measurement of pre-dose and control urinary taurine values can establish a baseline from which changes in urinary taurine can be monitored. It is important, however, to maintain animals on the same batch of diet as taurine in these is not consistent. Taurine excretion in control human subjects were within the limits of the published values, and similarly, they were also very variable. In particular there appeared to be a great deal of variation amongst female controls. This may have been partly related to the diet as those with the lowest urinary taurine levels were also vegetarians or ate little meat. However, the analysis of 'spot' urine samples suggests that there is less variation in taurine excretion within an individual than between individuals.

Urinary levels of taurine in the rat have been correlated with levels of taurine in liver [33] and in agreement with Hirai *et al.* [23] liver levels varied more than those in other tissues. This variation may account for the very different levels of taurine found in some isolated hepatocyte preparations. However, initial low liver values cannot account for the very low levels found in some subsequently isolated cells. The loss of taurine during isolation may be of concern in view of the reported protective properties of taurine in both isolated hepatocytes [34,35] and *in vivo* [36,37].

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