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Journal of Chromatography B, 754 (2001) 467–475

JOURNAL OF CHROMATOGRAPHY B

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Application of a high-performance liquid chromatographic assay for the neuromuscular blocker gallamine to analysis of rat plasma, muscle and microdialysate samples

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Received 13 July 2000; received in revised form 11 December 2000; accepted 5 January 2001

Abstract

A reliable high-performance liquid chromatographic method has been validated for determination of gallamine in rat plasma, muscle tissue and microdialysate samples. A C_{18} reversed-phase column with mobile phase of methanol and water containing 12.5 m*M* tetrabutyl ammonium (TBA) hydrogen sulphate (22:78, v/v) was used. The flow-rate was 1 ml/min with UV detection at 229 nm. Sample preparation involved protein precipitation with acetonitrile for plasma and muscle tissue homogenate samples. Microdialysate samples were injected into the HPLC system without any sample preparation. Intra-day and inter-day accuracy and precision of the assay were <13%. The limit of quantification was 1 μg/ml for plasma, 1.6 μg/g for muscle tissue and 0.5 μg/ml for microdialysate samples. The assay was applied successfully to analysis of samples obtained from a pharmacokinetic study in rats using the microdialysis technique. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Gallamine

1. Introduction

Gallamine (Fig. 1a), which is a synthetic substitute for curare, has been used as a non-depolarising neuromuscular blocking agent since the early 1950s [1]. Its clinical use has declined mainly because it is contraindicated in renally impaired patients [1], but gallamine is still used in pharmacological studies [2]. It is not metabolised [3], has negligible protein binding and exerts its neuromuscular blocking effects at plasma concentrations above 1 μg/ml [1,4]. Gallamine is employed as a model compound in our studies using microdialysis to investigate pharmacokinetics and pharmacodynamics of the neuromuscular blocker with continuous sampling from muscle tissue. HPLC methods with UV detection have been developed for quantification of gallamine by Shao et al. [5], Ramzan [1] and Mourier [6].

The method developed by Mourier [6] is not practical for routine analysis of pharmacokinetic samples since the purpose of the assay was to determine gallamine impurities. The methods of Shao et al. [5] and Ramzan [1] achieved a satisfac-

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PII: S0378-4347(01)00032-9

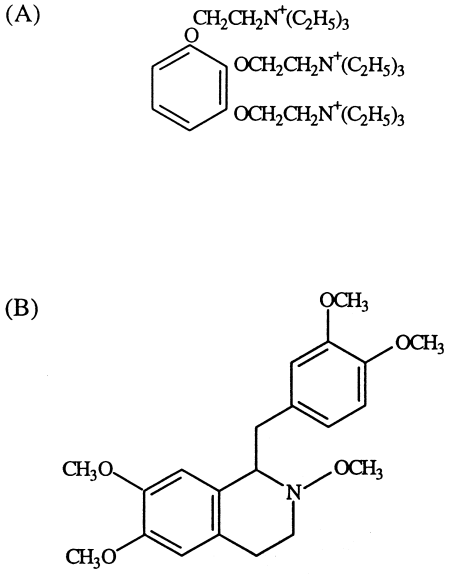

Fig. 1. Chemical structure of (A) gallamine and (B) internal standard laudanosine.

tory limit of detection. The HPLC assay developed by Ramzan [1] used small sample volumes (50 μl) and also employed a readily available internal standard. This method has been applied to the analysis of gallamine in rat plasma and has general applicability for human studies. However, its application to analysis of gallamine in muscle tissue and microdialysate samples has not been evaluated.

Microdialysis is a valuable tool to continuously sample the in vivo unbound concentrations of compounds in extracellular fluid. Details of this technique and its application to a variety of experimental settings, including pharmacokinetic and pharmacodynamic studies, have been reviewed elsewhere [7–9]. In summary, the in vivo microdialysis technique involves perfusion of the microdialysis probe implanted into the tissue of interest. The probe has a dialysis membrane at its tip. During perfusion, a fraction of compounds in the extracellular fluid is extracted into the probe allowing subsequent determination of the concentration of the analytes of interest in the perfusate. However, certain analytical considerations [10] are required to ensure the validity of concentration data obtained using this technique. One issue is assay sensitivity because of the relatively small volume of microdialysate sample (10–30 μl). Several studies which have employed the microdialysis technique to study drug distribution and metabolism have coupled the microdialysis system directly with analytical instruments to perform on-line analysis of microdialysate samples, which can overcome the issue of small sample volumes [11,12]. However, direct connection of the microdialysis system with the analytical instruments is not always applicable or feasible, and off-line analysis is used as an alternative option. In the present study, off-line HPLC has been used for the determination of gallamine in microdialysate samples.

Another issue is the in vivo recovery of compounds from tissue extracellular fluid using the microdialysis probe. In many cases, in vivo recovery is not complete which means that the concentration in the dialysate samples will be less than the concentration in the tissue extracellular fluid. Therefore, the quantification limit of the assay should be less than the lowest concentration expected in tissue extracellular fluid.

The objective of the present study was to optimise and validate an HPLC assay for determination of the neuromuscular blocker, gallamine, in rat plasma, muscle and microdialysate samples. The assay was applied to analysis of samples obtained from a pharmacokinetic study using the microdialysis technique to measure gallamine concentrations in muscle tissue.

2. Experimental

2.1. Chemicals and reagents

Gallamine as the triethiodide salt, DL-laudanosine (Fig. 1b, as internal standard, I.S.), and tetrabutylammonium (TBA) hydrogen sulphate were purchased from Sigma (NSW, Australia). HPLC-grade methanol and acetonitrile were purchased from Selby Biolab (NSW, Australia). Other chemicals were AR grade.

2.2. Chromatography

The HPLC apparatus consisted of a Shimadzu LC-10AS solvent delivery pump, a GBC autoinjector (LC1610), and a Shimadzu SPD-10A*vp* variable

wavelength UV–Vis detector (set at 229 nm) connected to a PC with PE Nelson Turbochrom professional (version 4.1) peak integration software. An Alltima® C_{18} analytical column (150×4.6 mm I.D., 5-μm particle size) preceded by an Alltima® C_{18} guard column (7.5×4.6 mm I.D., 5-μm particle size) from Alltech Associates (NSW, Australia) was used. The mobile phase consisted of methanol and water containing 12.5 m*M* TBA hydrogen sulphate (22:78, v/v), degassed under vacuum and filtered through 0.45-μm membrane filter (Millipore, Sydney Australia) prior to use. The flow-rate was 1 ml/min; the operating pressure was ~13 MPa. The HPLC system was housed in a temperature-controlled room (~20°).

2.3. Sample preparation

The following procedure was used for the determination of gallamine concentrations in plasma. Blank rat plasma spiked with a known concentration (range: 1–40 μg/ml) of gallamine standard solution in water or plasma (50 μl) obtained from the pharmacokinetic study was added with laudanosine (I.S.) solution (5 μl; 127.5 μg/ml in methanol) followed by acetonitrile (100 μl as a protein precipitant). The mixture was vortexed (15 s) and centrifuged (15 000 *g*, 2 min) in a microcentrifuge (Eppendorf microcentrifuge 5414S). The supernatant (100 μl) was then transferred to a borosilicate test tube and evaporated under a gentle stream of nitrogen (40°C). The resulting residue was reconstituted in mobile phase (100 μl) and an aliquot (30 μl) was injected into the HPLC system.

The concentrations of gallamine in muscle tissue were determined as follows. Muscle tissue (1 g) was homogenised in water (2 ml) using a homogeniser (Polytron, Kinematica, Switzerland). To homogenate of blank muscle tissue spiked with a known concentration (range: 1.6–39.1 μg/g) of gallamine standard solution in water or muscle tissue homogenate obtained from the pharmacokinetic study (100 μl) was added laudanosine solution (10 μl, 127.5 μg/ml in methanol). The same precipitation procedure as for plasma was employed and an aliquot (20 μl) was injected into the HPLC system.

The following procedure was used for the determination of gallamine concentrations in microdialysate samples. Gallamine standards (0.5–40 μg/ml) were freshly prepared in Krebs-Ringer bicarbonate solution (Krebs solution) as simulated extracellular fluid [13]. To gallamine in Krebs solution (30 μl), representing the volume of microdialysate samples obtained from rat muscle during the pharmacokinetic study or microdialysate samples obtained from in vitro or in vivo microdialysis experiments, was added internal standard (10 μl; 51.5 μg/ml in methanol) and an aliquot (20 μl) of this mixture was injected into the HPLC system without any additional sample preparation.

2.4. Linearity and quantification of gallamine

Calibration curves were generated by plotting the peak-area ratios of gallamine to laudanosine against the concentrations of gallamine. Unweighted linear regression was performed and the correlation coefficient was examined and accepted when $r^2 > 0.99$. Weighted linear regression with a weighting factor of $1/\text{concentration}^2$ and linear regression which takes into account potential errors in both dependent and independent variables [14] were also performed. Analysis of variance (ANOVA) was performed to evaluate the results using different linear regression approaches. Quantification of gallamine was achieved by interpolating the peak-area ratios on the calibration curves. The limit of quantification was defined as the lowest concentration of the calibration curves with accuracy and precision ≤15%, following replicate assays.

2.5. Assay recovery

The recovery of gallamine, following precipitation procedures, was assessed at four different concentrations in plasma (1, 6, 10 and 40 μg/ml) and three different concentrations in muscle tissue (1.6, 3.1 and 15.6 μg/g) with four replicates. The recovery was assessed as the percentage recovered from plasma and muscle tissue relative to the amount determined from an aqueous solution diluted with the mobile phase to a concentration corresponding to the final concentration of gallamine in the reconstituted samples after protein precipitation assuming that there is no loss of the drug during precipitation. The recovery of laudanosine, at the concentration used for the plasma and muscle assay, was assessed in six

replicates and the recovery was calculated in a similar manner as for gallamine.

2.6. Precision and accuracy

Intra-day precision and accuracy for the determination of gallamine from rat plasma and muscle tissue were determined using four replicates of spiked rat plasma at six different concentrations of gallamine (range: 1–40 μg/ml) and spiked muscle homogenate at five different concentrations of gallamine (range: 1.6–39.1 μg/g). For the determination of gallamine in microdialysate samples, intra-day precision and accuracy were examined using four replicates of gallamine in Krebs solution at seven different concentrations (range: 0.5–40 μg/ml). Inter-day precision and accuracy were assessed in plasma, muscle tissue and Krebs solution analysed on 4 days over a 2-week period.

The precision of the assay was expressed as percent coefficient of variation (C.V.). The accuracy of the assay was expressed as percent error and calculated as the percent difference of the actual concentration ($\overline{C}_{\text{actual}}$) from that of the mean value of the observed concentration ($\overline{C}_{\text{obs}}$) calculated from the calibration curve, following replicate assays:

$$\%\,Error = \frac{\overline{C}_{\text{actual}} - \overline{C}_{\text{obs}}}{\overline{C}_{\text{actual}}} \times 100\%$$

Accuracy and precision values of ±15% of the actual concentrations were considered to be acceptable.

2.7. In vitro microdialysis

The within-day stability of gallamine recovery from the microdialysis probe was studied by placing the probe (CMA/20, 4-mm membrane length, 20-kDa cut-off) in Krebs solution containing gallamine (10 μg/ml). The probe was continuously perfused at 2 μl/min with drug-free Krebs solution for 8 h. Microdialysate samples (30 μl) were analysed for the concentration of gallamine and the relative recovery of gallamine from the probe was calculated as the concentration of gallamine in the dialysate samples relative to that in the solution surrounding the probe.

2.8. Pharmacokinetic study in the rat using microdialysis technique

Plasma and muscle extracellular fluid concentration-time profiles of gallamine were determined after intravenous bolus administration of gallamine (6 mg/kg) to an urethane-anaesthetised rat. A total of eight serial blood samples (up to 6 h) were collected into heparinised tubes, and plasma was then harvested and frozen until analysis. A microdialysis probe (CMA/20, 10-mm membrane length, 20-kDa cut-off) was inserted into the left hind leg muscle using a needle guide and probe introducer for continuous sampling of the unbound concentrations of gallamine in the extracellular space of the hindlimb muscle. The probe was perfused with drug-free Krebs solution (flow-rate: 2 μl/min). Microdialysate samples (30 μl) were collected over the study period and refrigerated until analysis. Muscle tissue was excised at the end of the sampling period and stored frozen. Analysis of plasma, muscle and microdialysate samples was performed using the described procedures.

3. Result and discussion

A number of assays have been developed for the determination of gallamine. A dye-binding fluorescence method has been used in numerous pharmacokinetic studies of gallamine [15–18]. However, Shao et al. [5] reported that this analytical method produced significant variability due to impurities in the Rose Bengal dye, the complex preparation steps and the high fluorescence background.

The present HPLC assay was validated for determination of gallamine concentrations in rat plasma, muscle and microdialysate samples. The assay is a modification of a HPLC method developed by Ramzan, which used *d*-tubocurarine as internal standard [1]. During the preliminary work, the HPLC method developed by Ramzan was examined but no peak of *d*-tubocurarine appeared following the precipitation procedure, although direct injection of *d*-tubocurarine solution in water produced a peak similar to that reported by Ramzan [1]. Various precipitating agents [19] and types of C_{18} column were tested without success. Consequently, it was decided to select an alternate internal standard and

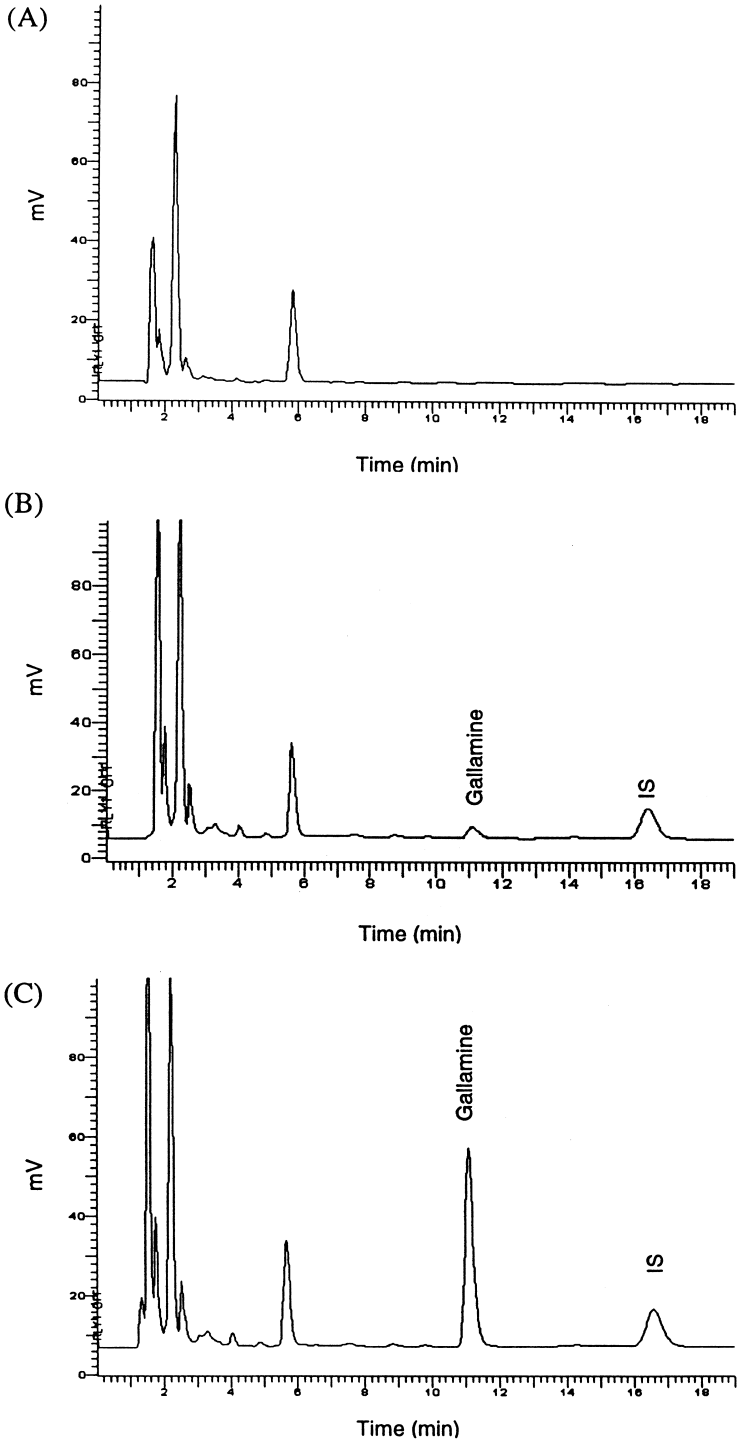

Fig. 2. Typical chromatograms of (A) blank rat plasma, (B) rat plasma spiked with gallamine (1 mg/ml), (C) rat plasma from pharmacokinetic study after 6 mg/kg i.v. bolus, assayed gallamine concentration was 20 mg/ml.

laudanosine was identified as a suitable internal standard using the Sigma–Aldrich chemical database (1995).

Further modifications to the assay of Ramzan [1] included optimising the concentration of organic modifier (methanol) and TBA hydrogen sulphate, and changing the precipitant agent (from a mixture of sodium tungstate and sulphuric acid to acetonitrile). The concentration of TBA hydrogen sulphate was increased in order to achieve optimum chromatographic conditions and separation of gallamine and internal standard. Lower concentrations of TBA hydrogen sulphate caused gallamine peak splitting when analysed in microdialysate samples, probably due to the interaction of TBA with various buffer ions in Krebs solution (since injection of gallamine in water gave a perfect chromatogram). The precipitant was changed to acetonitrile in this assay since the use of sodium tungstate and sulphuric acid mixture employed by Ramzan [1] caused a change of the chromatogram baseline that persisted long after the elution of the analytes of interest. Furthermore, an additional step was added in the sample preparation whereby the acetonitrile phase was evaporated and its residue was reconstituted in mobile phase. One advantage of using this protein precipitant is that the organic phase can be injected directly into the HPLC system. However, it was found in this study that direct injection of the acetonitrile into HLPC caused splitting of the peak of laudanosine.

The retention times of gallamine and laudanosine (internal standard) were 11 and 17 min, respectively. There were no interfering peaks with either gallamine or laudanosine from plasma (Fig. 2), muscle tissue (Fig. 3) or microdialysate samples. There was a peak which appeared at 5.5 min in all plasma samples, including blank plasma, that was subsequently found to have the same retention time as the anaesthetic agent (urethane) used in our studies. During assay development, it was found that the retention time of laudanosine was sensitive to temperature. Maintaining the HPLC system in a temperature-controlled environment led to reproducible retention times for both laudanosine and gallamine with % C.V. less than 2% ($n=50$).

Disruption of tissue structure during microdialysis probe insertion can lead to the possible release of endogenous compounds which have the potential to affect the chromatogram. Analysis of microdialysate samples at different times (15, 30, 45 and 60 min) after probe insertion into muscle tissue showed that endogenous compounds in the extracellular fluid reached equilibrium within ~30 min of the micro-

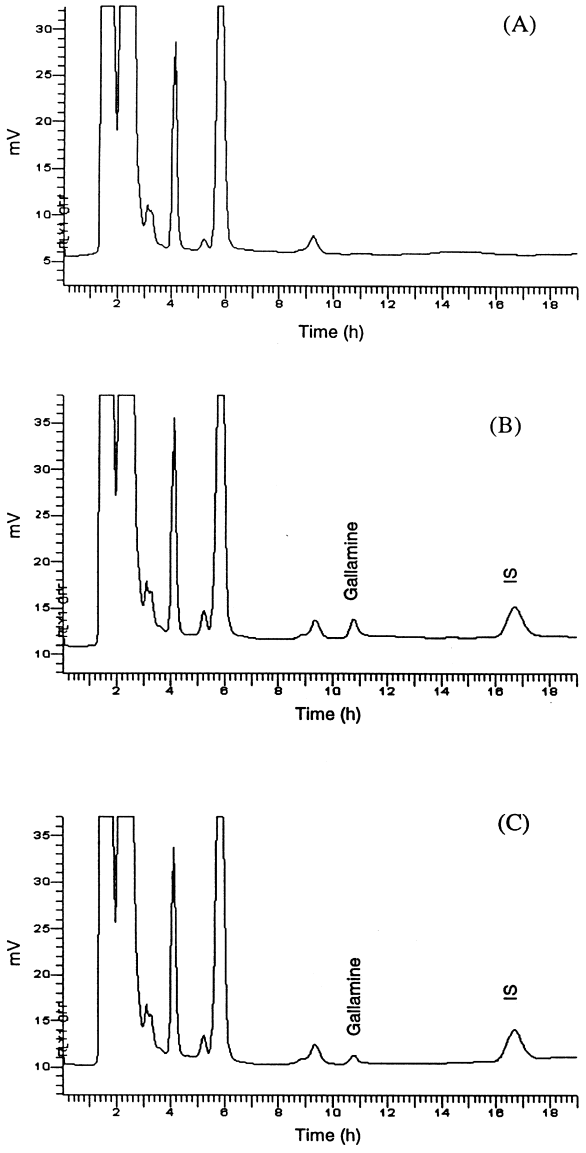

Fig. 3. Typical chromatograms of (A) blank rat muscle, (B) rat muscle spiked with gallamine (3 mg/g), (C) rat plasma from pharmacokinetic study after 6 mg/kg i.v. bolus, assayed gallamine concentration was 1.6 mg/g.

dialysis probe insertion, and there was no interference with either gallamine or internal standard.

Preliminary studies on the recovery of gallamine in the microdialysis experiments showed that a flow-rate of 2 μl/min with 15-min sampling intervals gave an optimal recovery of gallamine from the microdialysis probe. Under these conditions, 30-μl aliquots of microdialysate were collected and the present assay was validated to quantify gallamine in this volume of solution, with an injection volume of 20 μl.

A linear relationship was obtained between gallamine concentration and gallamine to internal standard peak-area ratios with the correlation coefficient in excess of 0.999 for plasma (1–40 μg/ml), microdialysate samples (0.5–40 μg/ml), and muscle homogenate (1.6–39.1 μg/g). The slope of the calibration curve was 0.136±0.004, 0.041±0.001 and 0.106±0.003 from plasma, muscle and microdialysate samples ($n=5$), respectively, estimated using unweighted linear regression. Analysis of variance shows there is no difference ($P>0.05$) in the calibration curve slopes using either unweighted linear regression, weighted linear regression with a weighting factor of $1/\text{concentration}^2$ or linear regression which takes into account potential error in both the dependent and independent variables. Therefore, for routine work unweighted linear regression was adopted. The intra-day (Table 1) and inter-day (Table 2) precision and accuracy for determination of gallamine concentrations in plasma, muscle tissue and microdialysate samples were within the defined acceptable range.

The recovery of gallamine was in the range of 90–98% ($n=4$) over the concentration range of 1–40 μg/ml and 1.6–15.6 μg/g from rat plasma and muscle tissue, respectively. The limits of quantification of gallamine were 1 μg/ml for plasma, 1.6 μg/g for muscle tissue and 0.5 μg/ml for microdialysate samples. The recovery of laudanosine from plasma and muscle tissue was 104±4 and 102±4%, respectively ($n=4$).

Table 1
Intra-day precision and accuracy of gallamine in rat plasma, muscle tissue and microdialysate samples[a]

Sample	Spiked concentration	Estimated concentration	Accuracy (%)	Precision (%)
Plasma	μg/ml	μg/ml		
	1.0	1.1±0.2	8.2	9.0
	2.5	2.6±0.1	4.4	4.5
	5.0	4.7±0.1	5.3	1.8
	10.0	9.6±0.5	3.9	4.5
	20.0	20.2±0.6	1.2	3.1
	40.0	40.0±1.2	0.0	3.0
Muscle tissue	μg/g	μg/g		
	1.6	1.4±0.1	11.2	5.6
	3.1	2.8±0.1	9.0	4.0
	7.8	7.7±0.3	1.3	3.8
	15.6	16.4±0.9	5.4	5.5
	39.1	38.8±1.3	0.8	3.4
Microdialysate	μg/ml	μg/ml		
	0.5	0.6±0.0	12.6	5.2
	1.0	1.0±0.0	4.1	2.4
	2.5	2.5±0.1	1.5	4.0
	5.0	5.0±0.1	0.9	1.8
	10.0	9.8±0.4	2.3	4.0
	20.0	20.1±0.4	0.6	2.0
	40.0	40.0±1.2	0.0	3.0

[a] $n=4$.

Table 2
Inter-day precision and accuracy of gallamine in rat plasma, muscle tissue and microdialysate samples[a]

Sample	Spiked concentration	Estimated concentration	Accuracy (%)	Precision (%)
Plasma	μg/ml	μg/ml		
	1.0	1.1±0.1	7.0	8.1
	2.5	2.5±0.2	0.4	6.7
	5.0	5.1±0.4	1.1	6.9
	10.0	9.8±0.9	1.7	8.8
	20.0	19.6±0.8	2.1	4.3
	40.0	40.2±0.4	0.6	1.1
Muscle tissue	μg/g	μg/g		
	1.6	1.4±0.3	12.4	12.6
	3.1	2.9±0.4	8.3	10.0
	7.8	7.3±0.5	6.0	6.4
	15.6	16.9±0.7	8.0	3.8
	39.1	38.7±1.1	1.0	2.8
Microdialysate	μg/ml	μg/ml		
	0.5	0.5±0.0	7.5	4.3
	1.0	1.0±0.0	4.2	4.9
	2.5	2.4±0.1	4.8	2.6
	5.0	5.0±0.2	0.1	5.3
	10.0	9.9±0.2	0.8	2.7
	20.0	20.5±0.5	2.6	3.1
	40.0	39.8±1.3	0.5	4.0

[a] $n=4$.

The assay described in this report has been used to measure gallamine concentration in rat plasma and muscle tissue, as well as from in vitro and in vivo microdialysate samples. The in vitro microdialysis recovery of gallamine from the probe was stable over 8-h study (42±2%). Fig. 4 shows the plasma and muscle extracellular fluid (corrected with microdialysis in vivo recovery data of gallamine) concentration-time profile of gallamine in an anaesthetised rat after the administration of an intravenous bolus of gallamine (6 mg/kg). Gallamine pharmacokinetic profiles were similar in muscle extracellular fluid and plasma; in contrast the concentration in muscle homogenate tissue was only 25% of the concentration in muscle extracellular fluid at the terminal sampling point. The present assay is a reproducible and reliable HPLC assay for determination of gallamine in microdialysate samples, rat plasma and muscle tissue.

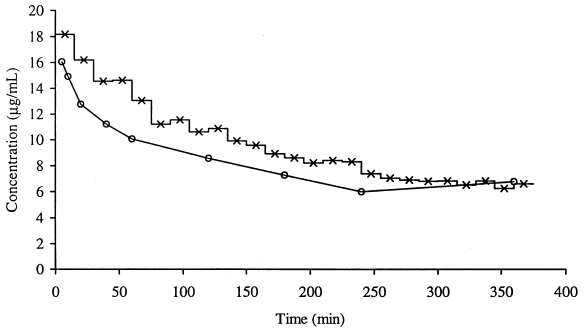

Fig. 4. Plasma (o) and muscle extracellular fluid (x) concentration versus time curves after i.v. bolus administration of gallamine (6 mg/kg) in an urethane-anaesthetised rat.

4. Conclusion

A sensitive and reliable HPLC assay was validated for the determination of gallamine from rat plasma, muscle and microdialysate samples. The assay is suitable for pharmacokinetic studies of gallamine in the rat using the microdialysis technique.

Acknowledgements

L. Sasongko is supported by Australian Development Scholarship. John Ray (Department of Clinical Pharmacology and Toxicology, St Vincent's Hospital, NSW, Australia) provided helpful comments in assay development.

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