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The effect of sodium fluoride preservative and storage temperature on the stability of cocaine in horse blood, sheep vitreous and deer muscle

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ABSTRACT

The *in vitro* stability of cocaine in horse blood, sheep vitreous humour (VH) and homogenised deer muscle is described. The stability of cocaine in horse blood was of interest because many toxicology laboratories utilise horse blood for the preparation of calibration and check standards and the latter are typically stored during routine use. The storage stability of cocaine in human VH and muscle has not been previously reported. In the absence of blank human VH and muscle, cocaine stability under varying conditions was demonstrated in animal tissues. Blood and VH were stored with and without addition of NaF at room temperature (RT), 4 °C and −18 °C for 84 days. Muscle homogenates were prepared in water, water/2% NaF, and phosphate buffer (pH 6.0)/2% NaF, and stored for 31 days at RT, 4 °C and −18 °C. Cocaine stability in human muscle obtained from cocaine positive forensic cases was assessed following storage at −18 °C for 13 months. Cocaine and benzoylecgonine (BZE) were extracted using SPE and quantified by GC-MS/MS. Cocaine was stable for 7 days in refrigerated (4 °C) horse blood fortified with 1 and 2% NaF. In the absence of NaF, cocaine was not detectable by day 7 in blood stored at RT and 4 °C and had declined by 81% following storage at −18 °C. At 4 °C the rate of cocaine degradation in blood preserved with 2% NaF was significantly slower than with 1% NaF. The stability of cocaine in horse blood appeared to be less than that reported for human blood, probably attributable to the presence of carboxylesterase in horse plasma. Cocaine stored in VH at −18 °C was essentially stable for the study period whereas at 4 °C concentrations decreased by >50% in preserved and unpreserved VH stored for longer than 14 days. Fluoride did not significantly affect cocaine stability in VH. The stability of cocaine in muscle tissue homogenates significantly exceeded that in blood and VH at every temperature. In preserved and unpreserved samples stored at 4 °C and below, cocaine loss did not exceed 2%. The increased stability of cocaine in muscle was attributed to the low initial pH of post-mortem muscle. In tissue from one human case stored for 13 months at −18 °C the muscle cocaine concentration declined by only 15% (range: 5–22%). These findings promote the use of human muscle as a toxicological specimen in which cocaine may be detected for longer compared with blood or VH.

1. Introduction

The poor stability of cocaine in human blood stored without the addition of preservative, i.e. fluoride, and without refrigeration is well documented [1–4]. The stability of cocaine in horse blood on the other hand has not been previously reported, despite this matrix being utilised extensively in toxicology laboratories in the UK and elsewhere for the preparation of blood calibration and check standards. Knowledge of cocaine stability in this matrix is of particular importance if standards are prepared in bulk and stored prior to use, as is typically the case for quality control standards which may be stored for up to 6 months during routine use. In humans cocaine is hydrolysed in the blood via two mechanisms: (1) enzyme hydrolysis mediated by butyrylcholinesterase (BuChE) forming ecgonine methyl ester (EME) [5], and; (2) spontaneous chemical hydrolysis at alkaline pH forming BZE [6,7]. BZE and EME are further hydrolysed in blood forming ecgonine [4]. Owing to residual BuChE activity, enzyme hydrolysis continues during sample storage [2,3]. Horse blood, in addition to containing BuChE [8], contains carboxylesterase (CE) [9], which is known to hydrolyse ester drugs such as cocaine [10–12]. Thus, the degradation of cocaine in stored horse blood would have an additional enzymatic pathway

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compared to human blood that might contribute to the breakdown of cocaine in this matrix.

As a consequence of the lack of esterase activity in VH, cocaine is detectable for longer in this matrix compared with blood [13–15]. Despite the potential forensic importance, data relating to the *in vitro* stability of cocaine in VH has not been reported. Although significant bacterial growth has been reported not to occur in VH [16–18], the addition of fluoride to this matrix has been shown to significantly increase the stability of ethanol, zopiclone and 6-acetylmorphine [19,20]. With the exception of ethanol analyses there are currently no recommendations on the utility of fluoride in preserving VH specimens for drug analysis. Muscle, like VH, lacks esterase activity [21] and a single *in vitro* study demonstrated an increase in the stability of cocaine in this matrix relative to blood [22]. The stability of cocaine in muscle has also been demonstrated by its detection in decomposed tissue at concentrations ranging from 0.003 to 1.5 mg/kg [23]. The *in vitro* stability of cocaine in muscle tissue stored under different conditions has not been previously reported.

This study examined the effect of storage temperature and sodium fluoride addition on cocaine stability in horse blood, sheep VH and deer muscle tissue homogenates. The study period spanned 84 days for blood and VH and 31 days for muscle tissue. Cocaine stability in authentic human muscle tissue homogenates containing sodium fluoride was also examined following storage at −18 °C for 13 months. Stability was assessed over time by measurement of the parent compound and its major metabolite, BZE.

2. Materials and methods

2.1. Specimens

Oxalated horse blood was obtained from TCS Biosciences (Buckingham, UK). Vitreous humour was obtained from sheep within 1 h of slaughter (Dunbia Abattoir, Carmarthenshire, UK). VH was aspirated by needle puncture through the sclera. Skeletal muscle was obtained from the hind quarter of freshly culled Sikah deer (*Cervus Nippon*; New Forest, Dorset, UK). All specimens were stored at −18 °C prior to experimental setup.

2.2. Specimen selection

Owing to restrictions imposed by the Human Tissue Act (1974), the use of human vitreous and human muscle tissue was not practical for this study. It is known that the vitreous gel of the various mammalian species is composed of essentially the same extracellular matrix components [24]. Thus, as with human VH, sheep VH predominantly consists of water and does not contain hydrolytic esterases. The sheep VH used in this study was also comparable in terms of pH; the pH of sheep VH following collection was pH 7.6 and in humans the pH of VH is reported to be pH 7.5 [25]. Pig eyes were initially collected for use in this study but yielded a very low volume of VH (~1–2 mL). The available volume of sheep VH was higher and more comparable to the volume available in humans (approximately 3–4 mL per eye). Skeletal muscle from Sikah deer was the best available specimen for this study because a very fresh sample could be obtained – it was possible to collect the tissue from the animal within 24 h of death. Further, the pH of deer muscle measured 24 h following death (pH 5.7 when homogenised with water) was comparable to that previously reported for early post-mortem human skeletal muscle tissue (pH 5.7–6.0) [26].

2.3. Experimental

Horse blood, sheep VH and deer tissue samples were spiked to achieve a final cocaine concentration of 0.4 mg/L (mg/0.5 kg for muscle). Although cocaine concentrations encountered in forensic cases vary widely, the chosen concentration falls in the mid range of those typically reported in forensic cases for blood and VH [14,15,27,28] and the mid to high range in muscle [29]. Blood samples were mixed on a rotary mixer for 30 min to ensure even distribution of the analyte throughout the sample. VH was vortex mixed thoroughly. Blood sample aliquots were prepared in triplicate at each of the three preservative conditions (0, 1 and 2% NaF) and at each of the three study temperatures, room temperature (RT, approximate range: 15–25 °C), 4 °C and −18 °C. Sufficient sample volume was stored in each aliquot (4 mL) to enable triplicate extraction at each analysis time (Table 1). Owing to restrictions on sample volume, sheep VH aliquots (1.5 mL) were prepared in duplicate at two preservative conditions (0 and 1.5% NaF) and stored at the same temperatures as for blood. Each VH sample was extracted in duplicate at each analysis time (Table 1).

Deer muscle homogenates were prepared with (1) water, (2) water containing 2% NaF, and (3) 1.0 M phosphate buffer (PBS, pH 6.0) containing 2% NaF. Following removal of all fat and connective tissue, muscle was segmented with scissors and subsequently homogenised at a 1:3 tissue to buffer ratio using a PowerGen 125 homogeniser (Fisher Scientific, Loughborough, UK). Multiple extractions of a single replicate were not carried out for muscle samples because in preliminary experiments the homogenates became more viscous and inhomogeneous as time of storage increased. To ensure that concentration differences observed between different storage conditions and analysis times were the result of differences in stability and not due to inconsistent sampling, muscle stability samples were prepared individually rather than in aliquots. Homogenate samples (2 g; equivalent to 0.5 g tissue) were weighed into a series of polypropylene centrifuge tubes and each spiked individually with 0.4 mg cocaine. Samples were vortex mixed thoroughly and set up in quadruplet at each preservative condition and stored at room temperature (approximate range: 22–25 °C), 4 and −18 °C. Each 2 g homogenate was extracted whole at each of the analysis times (0, 7, 14 and 28 days). Storage conditions and analysis times are presented in Table 1. The long-term stability of cocaine in muscle tissue homogenates was also assessed in authentic

Table 1
Storage conditions for spiked horse blood, sheep vitreous and deer muscle homogenate.

Matrix	Preservative condition	Study replicates (*n*)	Extractions per replicate (*n*)	Storage temperature	Analysis times (days)
Horse blood	Unpreserved	3	3	RT	0, 7, 14, 35, 54, 84
	1% NaF	3	3	RT	0, 7, 14, 35, 54, 84
	2% NaF	3	3	RT	0, 7, 14, 35, 54, 84
	Unpreserved	3	3	4 °C	0, 7, 14, 35, 54, 84
	1% NaF	3	3	4 °C	0, 7, 14, 35, 54, 84
	2% NaF	3	3	4 °C	0, 7, 14, 35, 54, 84
	Unpreserved	3	3	−18 °C	0, 7, 14, 35, 54, 84
	1% NaF	3	3	−18 °C	0, 7, 14, 35, 54, 84
	2% NaF	3	3	−18 °C	0, 7, 14, 35, 54, 84
Sheep VH	Unpreserved	2	2	RT	0, 7, 14, 21, 35, 54, 84
	1.5% NaF	2	2	RT	0, 7, 14, 21, 35, 54, 84
	Unpreserved	2	2	4 °C	0, 14, 21, 35, 54, 84
	1.5% NaF	2	2	4 °C	0, 14, 21, 35, 54, 84
	Unpreserved	2	2	−18 °C	0, 14, 21, 35, 54, 84
	1.5% NaF	2	2	−18 °C	0, 14, 21, 35, 54, 84
Deer muscle	Unpreserved (H_2O)	4	1	RT	0, 7, 14, 28
	2% NaF/H_2O	4	1	RT	0, 7, 14, 28
	2% NaF/PBS	4	1	RT	0, 7, 14, 28
	Unpreserved (H_2O)	4	1	4 °C	0, 7, 14, 28
	2% NaF/H_2O	4	1	4 °C	0, 7, 14, 28
	2% NaF/PBS	4	1	4 °C	0, 7, 14, 28
	Unpreserved (H_2O)	4	1	−18 °C	0, 7, 14, 28
	2% NaF/H_2O	4	1	−18 °C	0, 7, 14, 28
	2% NaF/PBS	4	1	−18 °C	0, 7, 14, 28

PBS: phosphate buffer (1 M, pH 6).